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<b>(54) Title:</b> ADIPOCYTE-SPECIFIC DNA SEQUENCES AND USE FOR THE PRODUCTION OF TRANSGENIC ANIMALS EXHIBITING ALTERED FAT METABOLISM  <b>(57) Abstract</b>  Regulatory DNA sequences are provided, which are obtained from the 5' flanking region of genes which are expressed primarily in differentiated adipose tissue. These DNA sequences are largely responsible for driving the expression of endogenous genes specifically in adipose tissue <i>in vivo</i> . The DNA sequences can be located in a region 5' of the gene, distinct from promoter sequences which provide a site for the initiation of transcription into DNA, or can be located within the region of the promoter itself. When operatively linked to a gene encoding a recombinant protein capable of exerting an effect on the metabolism of adipocytes, the DNA sequences of the invention can be used to produce transgenic animals which exhibit altered fat tissue metabolism. Depending upon the nature of the gene introduced in the animal or ancestor thereof at an embryonic stage, the transgenic animals are leaner or more obese than non-transgenic animals of the same species.		

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ADIPOCYTE-SPECIFIC DNA SEQUENCES AND USE FOR THE  
PRODUCTION OF TRANSGENIC ANIMALS EXHIBITING ALTERED FAT  
METABOLISM

5           This invention was made with Government support  
under National Institutes of Health Grant DK31405.  
The Government therefore has certain rights in this  
invention.

FIELD OF INVENTION

10           This invention relates to adipocyte-specific  
DNA sequences capable of directing expression of  
recombinant proteins specifically in adipose tissue  
in vivo, to an expression system which comprises the  
adipose-specific DNA sequence operatively linked to  
DNA sequences coding for a protein capable of  
15           exerting an effect on adipose tissue metabolism, and  
also relates to the use of the expression system in  
the production of transgenic animals which exhibit  
altered fat tissue metabolism. When the expression  
system is transgenically incorporated in an animal,  
20           the recombinant protein is expressed primarily in  
adipose tissue. The transgenic animals are either  
lean or obese compared to non-transgenic animals of

the same species, depending upon the nature of the DNA sequence operatively linked to the adipocyte-specific DNA sequence.

#### BACKGROUND OF INVENTION

5 Transgenic animals carry a gene which has been introduced into the germ line of an animal or an ancestor of the animal at an early stage in development. Wagner et al, Proc. Natl. Acad. Sci. USA, 78:5016 (1982). The ability to introduce new  
10 genes into the germ line of animals and thereby produce proteins outside of their normal environment and separated from their usual physiological control mechanisms has been extremely valuable for studying various aspects of gene expression. The technology  
15 also presents significant potential for improving various traits in animals, such as resistance to disease, reproductive rates and growth and lactation.

Much of the work involving transgene expression  
20 in animals has utilized the mouse as the experimental animal. For example, U.S. Patent No. 4,736,866, describes the generation of transgenic mice whose germ line cells and somatic cells carry an activated oncogene sequence introduced into the  
25 animal or the ancestor of the animal at a germ line stage. Numerous other genes have been introduced into mice in an effort to gain a better understanding of gene expression in animals. See,

for example, a review by Brinster and Palmiter, Harvery Lectures, 80:1-38 (1986) and Palmiter and Brinster, Annual Review of Genetics, 20:465-499 (1986).

5           More recently, gene transfer has been extended to domestic animals, including pigs, chickens, fish, cattle, rabbits and sheep. Pursel et al, Science, 244:1281-88 (1989). Many of the experiments in commercially important livestock have been designed to test the feasibility of introducing foreign growth promoting genes into the germ line of the livestock and thereby enhance growth performance. One approach that has received a great deal of attention is the introduction of growth regulating genes under the control of heterologous promoters into the germ line of the livestock, to allow long term production of the peptides in ectopic tissue.

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          The first attempt at applying the technology to domestic animals involved the introduction of a fusion gene including the mouse metallothionein (MT) regulatory/promoter sequence fused to the human growth hormone gene into the genome of pigs. Hammer et al, Nature, 315:380 (1985); Brem, Zuchhygiene, 20:251 (1985). Since that time, several other growth promoting genes, including rat, ovine and bovine growth hormone, human growth releasing-factor and bovine insulin-like growth factor have been

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introduced into the germ lines of a variety of domestic animals. Most of the transferred genes in commercially important livestock have been under the control of the mouse MT promoter.

5           The foregoing strategy has resulted in the stimulation of growth and enhancement of conversion of food to protein in pigs, Pursel et al, Science, 244:1281 (1989), which indicates that an important practical utility for the technology exists.  
10 Unfortunately, the procedures heretofore employed have also resulted in detrimental side effects on the general health of the transgenic animals. Id.

          In addition to the demand for improved rate and efficiency of body weight gain in commercially  
15 important livestock, there is also a strong demand in the agricultural industry for altered meat composition toward a leaner, less fat product consistent with medical advice that human beings reduce their consumption of animal fat. Although a  
20 side effect of the growth enhancing experimentation in transgenic animals has been a reduction in subcutaneous fat, the other deleterious side effects associated with the technology indicate that the prior technology is not a viable technique for  
25 providing leaner transgenic livestock. Other current strategies for growth regulation, including a shift toward leaner animals, include implantation or oral administration of natural or synthetic

steroids, injection of exogenous somatotropin (growth hormone) or growth hormone releasing factor, oral or parenteral administration of B-adrenergic agonists and immunoneutralization. For a review, see Beerman, Status of Current Strategies For Growth Regulation in ANIMAL GROWTH REGULATION, 377 (1989).

While various of these techniques have been successfully utilized to reduce carcass fat in animals, each involves the routine administration of the factors to individual animals and does not result in alteration of the germ line to provide a continuous source of leaner animals. Moreover, hormonal treatments have an effect on many tissues, not just the tissue whose composition is to be altered. Alternative approaches for use of gene manipulation to alter body composition toward less fat tissue are therefore indicated.

One of the advances that would significantly increase the likelihood of success of producing leaner strains of transgenic animals would be to place the genetic material under the control of a promoter or enhancer sequence specific for fat (adipose) tissue. Use of such a tissue specific control element should result in expression of the genes only in the tissue whose composition is to be altered. Such an adipocyte-specific control element could be used, not only in the agricultural industry to produce animals with reduced fat content, but

could also be utilized to alter fat metabolism in experimental animals. As an example, the fat-specific element could be used to alter the levels of endogenous genes that are thought to play key roles in the functioning of adipocytes, thereby allowing a better understanding of their roles in both adipose homeostasis and in disease states involving this tissue. The element could also be utilized in the development of small, organic pharmaceutical molecules which interfere with the binding of the protein factor to the adipose specific regulatory element, to thereby control transcription in a manner designed to combat regulatory defects associated with the disease state in fat tissue.

Prior research efforts have been directed to the elucidation of promoter/regulator sequences responsible for the expression of various proteins expressed primarily in adipocytes (fat-filled cells). For example, the promoter of the aP2 gene has been isolated and used as a model for the study of differentiation- and hormonally-linked gene regulation (Hunt et al, Proc. Natl. Acad. Sci. USA, 83:3786-3790 (1986); Phillips et al, J. Biol. Chem., 261: 10821-10827 (1986); Cook et al, Proc. Natl. Acad. Sci. USA, 85:2949-2953 (1988). AP2 is a novel gene product which is transcriptionally activated during adipocyte differentiation and is a member of



the lipid-binding protein family.) Sequences from the aP2 proximal promoter (-247 or -168 to +21) have been shown to direct differentiation-dependent expression of the bacterial chloramphenicol acetyltransferase (CAT) upon transient transfection into preadipocytes and adipocytes (Distel et al, Cell, 49:835-844 (1987); Yang et al, Proc. Natl. Acad. Sci. USA, 86:3629-3633 (1989); Cook et al, Proc. Natl. Acad. Sci. USA, 85:2949-2953 (1988); Christy et al, Proc. Natl. Acad. Sci. USA, 86:3629-3633 (1989)) and several regulatory elements that strongly influence this expression have been identified. These include an AP-1 site at -120, where a sequence-specific interaction between Fos-containing protein complexes and DNA was first demonstrated (Distel et al., Cell, 49:835-844 (1987); Rauscher et al., Cell, 52:471-480 (1988)). An additional positive-acting element at position -140 was shown to bind the transcription factor C/EBP in extracts from adipose cells and a distinct protein from preadipocyte extracts (Christy et al, Proc. Natl. Acad. Sci. USA, 86:3629-3633 (1989); Herrera et al, Mol. Cell Biol., 9:5331-5339 (1989)). Both the AP-1 and C/EBP binding sites have been shown to function positively in adipose cells and the AP-1 site is required for response of this promoter to cyclic AMP analogues (Herrera et al, Mol. Cell Biol., 9:5331-5339 (1989); Christy et al,

Proc. Natl. Acad. Sci. USA, 86:3629-3633 (1989).

The promoter for the adipose-specific gene product, adipsin, has also been studied in some detail.

5           None of these sequences have heretofore been shown to direct expression of genetic material in fat tissue in the animal. The current inability to direct expression directly to adipose tissue in vivo continues to be a major problem that has hampered  
10 the use of transgenic technology to regulate the fat metabolism of domestic animals.

          Accordingly, it is an object of the present invention to provide DNA sequences capable of directing the expression of recombinant proteins  
15 specifically in adipose tissue in vivo.

          Another object of the present invention is to provide an expression system comprising the adipose-specific DNA sequence operatively linked to a DNA sequence coding for a protein capable of altering  
20 adipose tissue metabolism.

          Another object of the present invention is to provide transgenic animals exhibiting altered fat tissue metabolism that can be used as animal models in the study of adipose homeostasis and disease  
25 states associated with fat tissue.

          A still further object of the present invention is to provide transgenic livestock with decreased adipose tissue content which are leaner than non-

transgenic animals of the same species.

#### SUMMARY OF THE INVENTION

These as well as other objects and advantages are achieved in accordance with the present invention which provides regulatory DNA sequences isolated from the 5' flanking region of genes which are expressed primarily in differentiated adipose tissue. These regulatory DNA sequences are primarily responsible for driving the expression of endogenous genes specifically in adipose tissue in vivo. The regulatory sequences can be located in a region 5' of the gene, distinct from promoter sequences which provide a site for the initiation of transcription into DNA, or can be located within the region of the promoter itself.

According to this invention, the adipose-specific DNA sequences are operatively linked to a DNA sequence coding for a recombinant protein capable of exerting an effect on the metabolism of adipocytes, and the thus-obtained expression system used to produce transgenic animals which exhibit altered fat metabolism. Depending upon the nature of the DNA sequence introduced in the animal or ancestor thereof at an embryonic stage, the transgenic animals are leaner or more obese than non-transgenic animals of the same species.

Since the regulatory sequences employed in the generation of the transgenic animals of the

invention are adipose-specific, the genes are expressed in adipose tissue, and expressed minimally, if at all, in other tissues, thereby reducing the potential for deleterious effects associated with non-tissue specific promoter/regulators. When a DNA sequence coding for a recombinant protein having a lipolytic or lipogenic effect on adipose tissue is introduced into the germ line of an animal, such as a mouse, under the control of an adipose-specific DNA sequence of the present invention, leaner or more obese animals, respectively, can be produced. Such animals are useful as models in the study of adipose homeostasis and the disease state associated with adipose tissue. When a DNA sequence coding for a protein having a lipolytic or anti-lipogenic effect on adipose tissue is introduced under the control of the adipose-specific element into the germ line of commercially important livestock, leaner strains of livestock can be produced.

#### **BRIEF DESCRIPTION OF THE DRAWING**

Figure 1A is a map of the plasmid pUC-CAT, which has been used to demonstrate the tissue specificity of the adipose-specific DNA sequences of the present invention.

Figure 1B is a restriction map of the 5' flanking region of the murine aP2 gene used to

construct the transgenes described herein. The genomic DNA was obtained from genomic clone aP2911, the preparation of which has been described. Hunt et al, Proc. Natl. Acad. Sci., 83: 3786-3790 (1986).

5        Figure 1C is a representation of the -168aP2, -247aP2, -1.7aP2 and -5.4aP2 constructs used to make transgenic mice. The open boxes represent aP2 sequences; the lines, CAT/SV40 sequences. The putative glucocorticoid regulatory element at -363  
10        nucleotides is represented by a solid bar, while the 512 base pair sequence containing the 5' adipose-specific enhancer is represented by a hatched box.

      Figure 2A comprises two chromatographs from CAT assays from two independently generated strains of  
15        247aP2CAT transgenic mice, 247aP2CAT 2, and 247aP2CAT 3<sub>a</sub>. The abbreviations used are: T, thymus; SC, spinal cord; SM, skeletal muscle; S, spleen; Lu, lung; B, brain; F, white fat; Li, liver; SG, salivary gland; BF, brown fat; H, heart.

20        Figure 2B is a chromatograph from the CAT assay from the 5.4aP2CAT 25 transgenic mouse. The abbreviations used are: T, thymus; SC, spinal cord; SM, skeletal muscle; S, spleen; Lu, lung; B, brain; F, fat; Li, liver; SG, salivary gland; BF, brown  
25        fat; H, heart.

      Figure 3 is a chromatograph illustrating the results of the RNAase protection assay of RNA isolated from the tissues of 5.4aP2adn transgenic

and non-transgenic control mice. A 430 bp fragment from the junction between the aP2 and adipsin sequences was cloned into the Bluescript vector and used to make the 475 bp probe. The X within the  
5 adipsin sequence represents an alternative splice site not present in the cDNA that is utilized in approximately 50% of the endogenous adipsin transcripts. A fragment of 406 nt is protected from the transcripts initiated in the aP2 sequences in  
10 the transgene, while the endogenous transcripts protect fragments of 352 nt and 276 nt.

Abbreviations utilized are N, non-transgenic; T, transgenic; S, spleen; BF, brown fat; F, fat.

Figure 4 illustrates the results of the CAT  
15 assays performed on extracts from 3T3-F442A adipocytes transiently transfected with 20  $\mu$ g of plasmid DNA, wherein the plasmids were a series of deletion constructs of -5.4 to -1.7 aP2CAT. The origin and orientation of the DNA fragments inserted  
20 at the Hind III site of the basal -63aP2CAT vector is indicated above each lane (e.g. -63/5.4-1.7 has the -5.4 sequence fused to the -63 sequence and the -1.7kb sequence at the 5' end of the construct) -63 is the basal vector alone.

25 Figure 5 illustrates the results of the RNAase T1 protection assay of RNA isolated from the tissues of a 512aP2CAT 2 transgenic mouse. A fragment spanning the junction between the aP2 promoter and

the CAT sequence 5' from 512aP2CAT DNA was subcloned into a Gemini vector and used to make the probe of 366 nt. Transcripts initiating from the aP2 sequences in the 512aP2CAT transgene protect a fragment of 294 nucleotides.

Figure 6 is a chromatograph illustrating the differentiation-dependent gene expression from the adipsin promoter DNA. Transfections were done with 10 $\mu$ g of each plasmid. Mock transfections included DEAE-dextran but no DNA. The pUCCAT-ADN plasmid contained adipsin sequences from -950 to +35 placed 5' to the CAT gene and no exogenous enhancer sequences. The pAE5 vector contained SV40 promoter sequences upstream of CAT and Akv enhancer sequences in the 3' position relative to the CAT gene. In pADN-CAT (-950)F, adipsin sequences -950 to +35 are in an inverted orientation relative to the CAT gene; in pADN-CAT (-950), the adipsin sequences are in the proper 5' to 3' orientation.

Figure 7 is a schematic map of the TAP vector described in Example 6. In the figure, the aP2 promoter is shown as the shaded bar; the PstI site (1519) is +21; the HindIII/EcoRI sites (1603) is -63 fused to -5.4 kb; and the XbaI site (2119) is -4.9 kb. The open bar represents the sequence from the BluescriptII+ vector; the hatched bar is from SV40 and contains the small t splice and polyadenylation signals; the closed bar is from aP2 promoter.

Coordinates are approximate.

Figure 8 is a schematic map of an expression system in accordance with the present invention, TAP MYOD. In the Figure, the open bar represents the  
5 sequence from Bluescript II+; the hatched bar is from SV40 and contains the small t splice and polyadenylation signals; the closed bar is from aP2 promoter and the circle bar comprises the DNA  
10 sequence coding for the muscle transcription factor, MYOD. Coordinates are approximate.

Figure 9 is a schematic map of an expression system in accordance with the present invention, TAP  $\alpha_2$  adrenergic. In the Figure, the open bar  
15 represents sequence from Bluescript II+; the hatched bar is from SV40 and contains the small t splice and polyadenylation signals; the closed bar is from aP2 promoter and the circle bar comprises the DNA  
sequence coding for the  $\alpha_2$  adrenergic receptor. Coordinates are approximate.

## 20 DEFINITIONS

As used in this application and claims, the terms recombinant protein and operatively linked have the following definitions:

25 Operatively linked - the linking of an adipose-specific DNA sequence to a DNA sequence coding for a desired protein so as to permit expression of that DNA sequence and production of that protein.



Recombinant protein - a protein or peptide coded for by a DNA sequence which is not endogenous to the native genome of the animal in whose adipose tissue it is produced in accordance with this invention or a protein or peptide coded for by a DNA sequence which, if endogenous to the native genome of the animal in whose adipose tissue it is produced, does not lead to the production of that protein or peptide in adipose tissue at the same level that the transgenic animal of this invention produces in adipose tissue.

#### DETAILED DESCRIPTION OF THE INVENTION

This invention relates to processes, DNA sequences, compositions of matter and transgenic animals. More specifically, the invention relates to the transgenic incorporation of one or more copies of an expression system or construct comprising an adipose-specific DNA sequence operatively linked to a DNA sequence coding for a recombinant protein capable of exerting an effect on adipose tissue metabolism. The construct is introduced transgenically into the germ line of an animal or ancestor thereof at an embryonic stage and the recombinant protein is expressed primarily in the adipose tissue of the animal. Depending upon the nature of the recombinant protein coded for by the DNA sequence, the transgenic animals are either

leaner or more obese than nontransgenic controls.

#### **Adipose-Specific Control Sequences**

The regulatory DNA sequences of the present invention, sometimes hereinafter referred to as  
5 adipose-specific DNA sequences, are primarily located in the 5' flanking region of genes that are expressed primarily in differentiated adipocytes. The DNA sequences can be enhancer sequences which, when operatively linked to a gene through a  
10 functional promoter, are capable of directing adipose-specific expression of the gene in the animal. Alternatively, the adipose-specific DNA sequences may be located within the promoter region.

In accordance with one embodiment of the  
15 present invention there is provided a functional, adipose-specific control sequence isolated from the murine adipocyte P2 gene. The aP2 upstream control element is an enhancer sequence, comprising about 512 nucleotides, and is contained in the 5' region  
20 of the murine aP2 gene, at about nucleotides -5.4kb to -4.9kb (an EcoRI XbaI restriction fragment). When linked to a DNA coding sequence through a functional promoter, this 512 base pair sequence is capable of directing the adipose-specific expression  
25 of the gene in vivo. Larger regions of the 5' flanking regions can similarly be used, as long as the 512 bp are included in the sequence.

It will be appreciated by those skilled in the art that variations in the 512 base pair sequence may exist due to genetic polymorphisms or cell-mediated modification of the genetic material.

5 Furthermore, the DNA enhancer sequence can be manipulated by genetic techniques to produce a slightly different sequence of bases. Such sequences are within the scope of the present invention where the ability of the sequence to

10 direct fat-specific expression in the animal is retained. For example, smaller fragments or deletions of the 512 base pair sequence may be useful in directing fat-specific expression in vivo. A method for preparing and screening smaller

15 fragments is described in the examples herein. Methods of preparing deletion mutations are conventional in the art; such mutants can similarly be screened as discussed herein.

Also provided is an adipose-specific DNA

20 sequence isolated from the 5' region of the murine adipsin gene. The adipsin adipose-specific sequence is located at about nucleotides -114 to +35 of the murine adipsin gene, a region which also includes the promoter for the adipsin gene. When linked to a

25 gene in the proper orientation, this 149 base pair sequence is also capable of directing fat specific expression of genetic material in vivo. For promoter sequences such as the murine adipsin

sequence, a heterologous enhancer can be employed to increase the level of adipose-specific expression. Examples of suitable enhancer sequences include, but are not limited to, the Akv viral enhancer, the SV40 viral enhancer, the murine sarcoma virus enhancer and the aP2 enhancer element.

Additional adipose-specific DNA sequences can be isolated from other adipose differentiation-linked genes using the methodology described herein, which will similarly be useful in the generation of the transgenic animals with altered fat metabolism. Examples of other adipose differentiation linked genes include, without limitation, the genes encoding glycerophosphate dehydrogenase (GPD), and stearoyl CoA reductase, hormone sensitive lipase and lipo-protein lipase.

#### Suitable Promoters

The expression systems or constructs of the present invention comprise an adipose specific DNA sequence operatively linked to a DNA sequence coding for a recombinant protein capable of exerting an effect on the metabolism of adipose tissue. Where the adipose-specific sequence is an enhancer sequence, the operative linkage is through a functional promoter.

Promoter DNA sequences suitable for use with the enhancer sequences of the present invention

include both homologous and heterologous promoters.  
For sequences comprising the 512 base pair murine  
aP2 enhancer sequence, a suitable homologous  
promoter is a basal promoter comprising nucleotides  
5 -63 to +21 of the murine aP2 gene.

The enhancer sequences of the present invention  
can also be linked to the DNA coding sequence  
through a heterologous promoter. Suitable  
heterologous promoters may include promoters which  
10 are functionally responsive to environmental  
variations in the concentrations of metals and/or  
steroid hormone compounds. These inducible  
promoters include promoter sequences which are  
naturally associated with the transferrin gene of  
15 chickens, the steroid/hormone responsive gene  
associated with the ovalbumin gene of chickens and  
the metal and steroid hormone responsive promoter of  
the mouse metallothionein genes. Sequences which  
are neither hormone nor metal responsive which can  
20 be used in accordance with the present invention  
include the truncated SV40 promoter, liver  
promoters, immunoglobulin gene promoters and heat  
shock promoters.

Of the heterologous promoters, the truncated  
25 SV40 or metallothionein promoter is preferably  
employed.

**DNA Coding Sequences**

5 The DNA coding sequences to be expressed fat-specifically in transgenic animals in accordance with the present invention are DNA sequences which  
10 code for a recombinant protein capable of exerting an effect on the growth and/or metabolism of fat cells. The DNA coding sequences can either be endogenous or exogenous to the animal whose metabolism is to be altered and the effect of the  
15 genes can be either direct or indirect. Genomic or cDNA can be used.

20 The DNA coding sequences to be expressed in adipose tissue of the transgenic animals include sequences coding for recombinant proteins which  
25 exhibit a lipolytic effect on adipose cells. Such proteinaceous materials are characterized by an ability to decompose or break fat down. The expression of proteins or polypeptides in adipose tissue that have a lipolytic effect on fat tissue  
30 will have a leaning effect on transgenic animals bearing the transgene. Similarly, proteins having an anti-lipogenic effect can result in leaner animals.

35 The DNA sequence can alternatively code for proteins that have a lipogenic effect on adipose cells, characterized by an ability to cause fat formation. Expression of proteinaceous materials in fat tissue which exert a lipogenic effect will

result in increase fat formation, resulting in animals which are obese. Anti-lipolytic factors, which cause a decrease in fat decomposition, would also be expected to result in more obese animals.

5           Numerous factors which have an effect on adipose tissue metabolism are known. These factors can broadly be categorized into several different classes of proteinaceous materials, including hormone receptors, oncogenes, ligands, enzymes and  
10 transcription factors. Examples of each of these classes and materials are provided below.

#### Hormone Receptors

Genes encoding hormone receptors that can be used in accordance with the present invention  
15 include those encoding the alpha and beta adrenergic receptors, the growth hormone receptor and wild type and mutant insulin receptors. The beta adrenergic receptors, including  $\beta_1$ ,  $\beta_2$  and  $\beta_3$ , are transducers of catecholamine action in fat cells and have a  
20 lipolytic effect on these cells. Gilman et al, "The pharmacological basis of therapeutics", MacMillan Publishing Co., New York, Chapter 8: 145-180 (1985). An overexpression of this receptor in fat tissue will have a leaning effect in animals bearing this  
25 transgene.

The  $\alpha_2$  adrenergic receptor, which also binds catecholamines, has an anti-lipolytic effect on

adipose cells (Berlan et al, Eur. J. of Clin. Invest., 15: 341-348 (1985); Galitzky et al, Eur. J. Of Clin. Invest., 18: 587-594 (1988)) and its overexpression in fat cells will cause a decrease in the rate of triglyceride breakdown. This will result in an decrease in fat breakdown and the animal will be obese.

The growth hormone, acting through its specific receptor, causes fat breakdown. Expression of the gene encoding the growth hormone receptor causes increased lipolysis in adipose tissue, which will have a leaning effect in the animals bearing this transgene.

The insulin receptor is responsible for the lipogenic, anti-lipolytic effects of insulin. The wild type insulin receptor increases lipid accumulation when overexpressed in fat. Overexpression of the wild type insulin receptor in fat will lead to an increased fat accumulation resulting in an obese animal. Alternatively, there are known mutations in the insulin receptor that inactivate its function and also inactivate other wild-type receptor molecules present in the same cells. Overexpression of these "dominant-negative" insulin receptors will result in a decreased sensitivity of fat cells to insulin, resulting in animals that have a decreased level of fat storage. Such animals will be lean.



### Oncogenes

Various oncogenes or protooncogenes can interfere with fat cell differentiation and fat storage in cultured adipocytes. Examples of such  
5 oncogenes include c-myc (Freytag, S. O., Mol. Cell Biol., 8: 1614-1624 (1988)) and the SV40 large T antigen (Cherington et al, Mol. Cell Biol., 8: 1380-84 (1988) or c-ski (Sutrave et al, "Genes and Development", in press (1990)). These genes can be  
10 linked to the upstream adipose-specific control sequences of the present invention and interfere with the ability of fat cells to differentiate or carry out their lipid accumulation pathways. Overexpression of these gene products may or may not  
15 result in tumor formation.

### Ligands

There are a large number of polypeptides that influence adipose tissue to store or breakdown triglycerides. The genes for these ligands include,  
20 but are not limited to: insulin (lipogenic); growth hormone (lipolytic); tumor necrosis factor (lipolytic); glucagon (lipolytic); and adrenal corticotropic hormone (lipolytic). Expression of these secreted peptides from adipose cells should  
25 cause a higher level of expression present in adipose tissue than in the systemic circulation, resulting in greater effects in adipose tissue than in other tissues.

**Enzymes**

In accordance with the present invention, it is also possible to directly overexpress several intracellular enzymes or extracellular enzymes that are involved in pathways of accumulation or breakdown of triglycerides in adipose cells, Examples of such enzymes include without limitation: hormone sensitive lipase (lipolytic); stimulatory G protein (lipolytic); inhibitory G protein (lipogenic); fatty acid synthetase (lipogenic); lipoprotein lipase (lipogenic) and acetyl CoA carboxylase (lipogenic).

**Transcription Factors**

It is also possible to interfere with adipose tissue development by expression in fat cells of a transcription factor specific for another developmental lineage, such as muscle. By expression of MYOD1, a muscle transcription factor, it is possible to interfere with fat development and alter this development to the muscle lineage. An animal bearing this transgene will be leaner and should also be more muscular than non-transgenic animals of the same species.

Other proteinaceous materials capable of exerting an effect on the metabolism of fat cells will be apparent to those skilled in the art and are considered within the spirit and scope of the

present invention.

#### Expression Systems

The constructs or expression systems of the present invention are prepared by operatively  
5 linking the DNA coding sequence to the adipose-specific DNA sequence. For enhancer sequences, such as the murine aP2 enhancer, the operative linkage of the enhancer to the structural gene is through a functional promoter. Generally, the adipose-  
10 specific upstream enhancer sequence is placed upstream of the promoter sequence, and can be linked in either a 5' to 3' or inverted (3' to 5') orientation. Alternatively, where the adipose-specific DNA sequence is itself located with a  
15 promoter region, the adipose-specific sequence is operatively linked directly to the functional gene in a '5 to 3' orientation.

Preferably, the expression system or construct of this invention also includes a 3' untranslated  
20 region downstream of the DNA sequence coding for the recombinant protein. Among the 3' regions which are preferably employed are sequences that provide mRNA splice and polyadenylation signals. Such sequences may be derived, for example, from the SV40 small t  
25 antigen or other 3' untranslated sequences well known in the art.

Methods for producing the above-described expression systems are well known. For example, various ligation techniques employing conventional linkers, restriction sites, etc., may be used to good effect. Preferably, the expression systems are prepared as part of larger plasmids. Such preparation allows the cloning and selection of the correct constructions in an efficient manner, as is well known in the art. Most preferably, the expression systems are located between convenient restriction sites on the plasmid so that they can be easily isolated from the bulk plasmid sequences.

#### Transgenic Animals

The transgenic animals of the present invention are obtained by introducing the linearized construct or expression system into the animals at an embryonic stage. The animals which can be usefully employed in accordance with the present invention are not particularly limited, and can include laboratory animals such as mice, rats, guinea pigs, rabbits, monkeys and domestic livestock, including cattle, pigs, sheep and chickens. The adipose-specific DNA sequence employed in the expression system need not be endogenous to the animal whose germ line is to be altered. Thus, for example, the murine aP2 enhancer sequence can be utilized to direct the adipose-specific expression of a

transgene in other animals, such as pigs. For practical reasons, it may be preferable to use a control sequence endogenous to the animal whose germ line is to be altered. In view of the known  
5 sequence homology of adipose differentiation-linked genes, such adipose-specific sequences can readily be obtained using the murine sequences described herein as hybridization probes.

10 In addition, a particular adipocyte-specific sequence may be more effective in one animal than others. One of skill in the art can readily make such choices by following the teachings of the invention.

Techniques for introducing transgenes into  
15 genetic material are known and have become conventional in the art. The following references, which describe procedures that can be used in accordance with the present invention, are hereby incorporated by reference. Evans et al, U.S. Patent  
20 No. 4,870,009, Miller et al, Journal of Endocrinology, 120:481-488 (1989); Hammer et al, J. Anim. Sci., 63:269-278 (1986) and Hammer et al, Cold Spring Harbor Symposium, Quant. Biol., 50: 379-387 (1985).

25 Briefly, a fertilized egg is obtained from the animal and the constructs are introduced into the fertilized egg in linear form. Several methods have successfully been employed to introduce foreign DNA

into the genetic material of mice, including microinjection of eggs, retrovirus infection of embryos, embryonic transfer of stem cells in blastocysts (Pursel et al, Science, 244: 1281  
5 (1989), and spermatzoa-mediated transfer of DNA (Lavitrano, Cell, 57: 717-723 (1989). For domestic animals, such as livestock, microinjection of the constructs into embryos is the method of choice. Preferably, the DNA is injected into the pronucleus  
10 of one cell eggs or the nucleus of two cell eggs.

Visualization of the nuclei of some animals, such as rabbits and sheep, can be aided through the use of interference-contrast (IC) microscopy. For less visible nuclei, such as those obtained from  
15 pigs and chickens, interference contrast microscopy alone will generally be insufficient. In such instances, visibility for microinjection can be enhanced by first centrifuging the eggs, followed by I-C microscopy. Wall et al, Biol. Rep., 32:645  
20 (1985).

The fertilized eggs having the fusion gene are then implanted into a host female of the species from which the fertilized eggs were obtained, so that the host female gives birth to a transgenic  
25 animal that develops from the fertilized egg. Usually, about 20-40% of the animals developing from the injected eggs contain at least one copy of the cloned fusion gene in somatic tissues and the

transgenic animals usually transmit the gene through the germ line to the next generation. For those species which give birth to more than one offspring, non-transgenic siblings provide an excellent control for determination of altered fat metabolism and fat tissue content.

The living transgenic animals and their progeny producing the metabolism-altering proteinaceous materials (which for purposes of this invention are considered the equivalent of the first generation transgenic animal) are continuous sources of leaner or more obese animals.

Offspring are tested for the presence of the transgene using techniques well known to those skilled in the art. For example, total nucleic acid can be extracted from a piece of the tail of the offspring and used for DNA dot hybridization according to the technique of Southern (J. Mol. Biol., 98: 503 (1975)) to determine which animals carry the transgene. Labeled DNA or RNA probes complementary to a portion of the gene are employed in the hybridization and animals that yield hybridization signals above the background are detected. Alternatively, polymerase chain reaction using appropriate probes can be employed to detect the presence of the transgene in the sample of total nucleic acid.

Altered fat metabolism in the transgenic animals resulting from the expression of the transgene in adipose tissue can be detected in a number of different ways. In rodents, such as mice, altered fat metabolism can be detected by measuring adipose cell volume and also by measuring fat pads. The latter technique involves sacrificing the animals and then removing and weighing the fat pads (gonadal, peri-adrenal, subcutaneous and abdominal). Other analyses, including determination of rates of fatty acid synthesis and/or lipolysis, determination of blood-lipid composition, in vivo, assays of  $^{14}\text{C}$ -labeled glucose incorporation into adipose tissue and adrenergic agonist and antagonist treatments of the animals, may also be employed.

In livestock, such as pigs and sheep, altered fat tissue metabolism can be detected by measuring back fat content in accordance with various techniques known in the art. For example, metabolism in the rump region has previously been shown to be representative of back fat. Subcutaneous back fat content can therefore be determined taking biochemical measurements from transgenic and control animals of the same species. In accordance with this method, tissue can be removed surgically from the rump region of the offspring and subjected to enzyme assays as previously described. The rate of fatty acid



synthesis in adipose tissue samples can be determined as described by Sinnett-Smith and Woolens, Anim. Prod., 45:75-80 (1987). Rates of lipolysis can determined by measuring the rates of glycerol release from adipose tissue. Sinnett-Smith and Wollens, Anim. Prod., 47:263-270 (1988). See also, Sinnett-Smith and Woolens, Int. J. Biochem., 21:535-540 (1989).

Other techniques for assessing altered adipose tissue metabolism include ultrasonic and direct back fat measurement. Magnetic resonance imaging can also be employed. Real time ultrasound is particularly preferred, as it causes less stress to the animals than other techniques which require surgical intervention and has become a reliable technique for determining fat content, particularly in the pork industry. Terry et al, J. Anim. Sci., 67:1279-1284 (1989); Mersmann, J. Anim. Sci., 54: 268 (1982).

#### 20 Utility

The adipose-specific DNA sequences of the present invention are useful in the generation of transgenic animals exhibiting altered fat tissue metabolism. In addition, the adipocyte specific enhancers can be used for the development of drugs to alter fat cell function and treat obesity.

Enhancers generally function by serving as binding sites for specific nuclear proteins. The function of the fat-specific enhancer aP2, for example, is mediated by the binding of nuclear proteins important in the regulation of this (aP2) gene and presumably, other fat cell-specific genes as well.

To identify new therapeutics, the adipocyte enhancer can be further dissected by ligating subfragments of the 512 bp enhancer to the minimal (-63 to +21) aP2 promoter or a heterologous promoter such as the SV40 promoter. These smaller fragments can then be used to study interactions with specific nuclear proteins by DNaseI footprinting techniques or a "gel-retardation" electrophoresis assay. The functional significance of these interactions with specific proteins can be analyzed by making small mutations that prevent individual proteins from binding. Once a protein factor (or factors) is identified that is important in the enhancer function, the DNA-protein interaction assays of interest (footprinting or gel-retardation) can be used as a high through-put screen for small molecules that inhibit these interactions. The ability to regulate fat cell gene expression specifically will depend upon finding a nuclear protein with specificity for adipose cells. This could be a protein found only in adipose cells or a

protein with a wider tissue distribution that has a modification (covalent or non-covalent) specific for this tissue.

5 The transgenic animals of the present invention, exhibiting altered levels of endogenous gene products, can be used as models to obtain a more complete understanding of the role of such genes in adipose tissue, both in adipose homeostasis and in the disease states involving this tissue.

10 For example, it is known the adipsin is deficient in several animal models of obesity. Flier et al, Science, 237: 402-405 (1987); Platt et al, Proc. Natl. Acad. Sci., 86: 7490-7494 (October 1989); Wilkison et al, J. Biol. Chem., 265: 477-482

15 (January 1990). To gain a better understanding of adipsin's physiological role in obesity, the adipsin gene can be overexpressed in adipose tissue of obese mice in accordance with the present invention. The mice will have adipsin sequences (cDNA and/or

20 genomic) under the control of the aP2 enhancer, which is not regulated by the obese state of the animal. The aP2 adipsin transgenic animals will show increased levels of adipsin expression in adipose tissue due to expression of the transgene.

25 The transgenic animals of the invention can also be used to test the efficacy of pharmaceutical agents designed to control disorders associated with adipose tissue.

When the invention is applied to commercially important livestock, the animals can be used as continuous sources of feed animals which exhibit reduced fat content.

5     Deposits

          A first construct according to this invention, prepared in accordance with Example 7 herein is exemplified by a culture deposited in the American Type Culture Collection, Rockville, Maryland, on  
10     September 25, 1990, and there identified as pTAP-MYOD. It has been assigned ATCC accession No. 40895.

          A second construct according to this invention, prepared in accordance with Example 8 herein is  
15     exemplified by a culture deposited in the American Type Culture Collection, Rockville, Maryland, on September 25, 1990, and there identified as pTAP- $\alpha$ 2. It has been assigned ATCC accession No. 40894.

          The present invention will be more readily  
20     understood from the following specific examples.

EXAMPLE 1

          This example demonstrates that a murine aP2 DNA enhancer sequence conferring adipose-specificity in vivo is located between -5.4 and -1.7kb, in the 5' flanking region of the murine aP2 gene. This  
25     example also demonstrates that the aP2 promoter

region previously shown to function in a differentiation dependent manner in adipocytes in culture (-168 to +21, Distel et al, Cell, 49: 835-844 (June 19, 1987)) is insufficient to drive expression in vivo.

#### Preparation Of Constructs

The aP2 sequences used in the preparation of the constructs were isolated from aP2 genomic DNA from lambda phage clone aP2911, available from Dr. Bruce Spiegelman of the Dana-Farber Cancer Institute, Boston, MA. A restriction map of the murine aP2 5' flanking region used to construct the transgenes is illustrated in Figure 1B. See also, Hunt et al, Proc. Natl. Acad. Sci., 83: 3786-3790 (1986).

Four constructs, containing either 168 base pairs (bp), 247bp, 1.7 kilobases (kb) or 5.4kb of the aP2 5' flanking region of the murine adipocyte P2 gene linked to chloramphenicol acetyltransferase gene sequences and the SV40 small t antigen splice site and polyadenylation signals, were obtained. Chloramphenicol acetyltransferase (CAT) is a bacterial gene encoding an enzyme which is widely used in the study of gene regulation. The expression of this enzyme in mammalian cells provides a sensitive, albeit indirect, assay for the transcriptional activity of particular DNA fragments

in transient transfections.

The vectors used for the 168aP2CAT and 247aP2CAT transgenes have previously been described. See, Distel et al, Cell, 49: 835-844 (June 19, 1987). Briefly, the plasmid containing -168 to +21 of the murine aP2 promoter (BclI-PstI restriction fragment) was prepared by placing the -168 to +21 sequence upstream of the bacterial chloramphenicol acetyl transferase gene of the plasmid pUC-CAT.

The pUC-CAT plasmid, which is illustrated in Figure 1A, was prepared from the vector pUC13 (part of the Messing series of pUC plasmids) and includes an insert containing the CAT coding region and SV40 3' processing sites, i.e., the intron and polyadenylation signal sequence. The latter DNA was isolated from pSVOCAT, described in Gorman et al, Mol. Cell Biol., 2: 1044-51 (1982). The pUC13 vector was isolated as a SmaI and EcoRI cut fragment; the insert was prepared by cutting pSV0 with HindIII, converting the thus obtained fragment with SmaI and then partially digesting with EcoRI to release the appropriate fragment (Ca. 2.4 kb). The vector and the insert were then ligated together and the product pUC-CAT (ca. 5.1 kb) isolated. A 190 base pair (BclI-SmaI) fragment was inserted into pUC-CAT isolated as a 5.1kb SmaI and partial Bam HI digestion.

The plasmid containing -247 to +21 of the murine aP2 promoter was prepared by placing the -247 to +21 PstI restriction fragment upstream of the CAT gene of the same PUC-CAT plasmid.

5           The -1.7aP2CAT transgene was prepared by ligating a -1.7kb EcoRI to +21 bp PstI fragment of the aP2 gene into pUC-CAT. pUC-CAT was prepared for cloning by cutting with HindIII, filling in the ends with Klenow, partially digesting with PstI and then  
10           isolating the 5.1kb vector. The -1.7kb to +21 insert was obtained from the clone 4.1aP2. (4.1aP2 was obtained by subcloning a 4.1kb EcoRI fragment, isolated from the genomic phage clone aP2911, into pBS (Stratagene, LaJolla, CA). The 4.1kb fragment  
15           contains the aP2 gene and -1.7 of 5' flanking region). The insert was obtained by cutting 4.1aP2 with EcoRI, filling in the ends with Klenow, cutting with PstI and then isolating the 1.7kb promoter. The thus prepared vectors and inserts were then  
20           ligated and appropriate clones isolated. The resulting plasmid, -1.7aP2CAT, was about 6.8kb, and exhibiting the following structure:

                  -1.7kb       -168       +21  
HindIII/EcoRI.....BclI.....PstISalIXbaIBamHISmaI-CAT-  
25           SV40....pUC13....HindIII/EcoRI.

The / indicates that the two sites were joined by blunt end ligation of Klenow filled-in restriction sites.

-5.4aP2CAT was generated by first  
 5 reconstructing the -5.4kb to +21 aP2 promoter into Bluescript IISK+ (pSK, Stratagene, La Jolla, CA), releasing the insert as a SmaI to HindIII fragment and then inserting the thus released fragment into SmaI and HindIII cut pUC-CAT. Initially, the 1.7kb  
 10 EcoRI to PstI fragment from the aP2 genomic clone was inserted into EcoRI and PstI cut pSK to generate -1.7aP2. A 3.7kb EcoRI fragment (-5.4 to -1.7kb) from the same genomic clone was then inserted into EcoRI cut -1.7 aP2 and the correct orientation was  
 15 isolated to generate -5.4 aP2pSK. The entire -5.4kb to +21 fragment was then isolated as a HindIII to SmaI fragment from -5.4pSK and inserted into HindIII and SmaI cut pUC-CAT to generate -5.4aP2CAT:  
 20                   -5.4       -1.7    +21  
 HindIIIEcoRVEcoRI....EcoRI...PstISmaI...CAT...SV40..  
 .pUC13...HindIII

#### Generation Of Transgenic Mice

Transgenes containing either -168 to +21 (-168aP2CAT) or -247 to +21 (-247aP2CAT) base pairs of  
 25 the promoter and 5' non-coding region linked to CAT sequences and the SV40 small t antigen splice and



polyadenylation signals were purified from the plasmid vectors by restricting the plasmids and isolating the DNA fragments for microinjection from the bulk of the plasmid sequences either by  
5 extraction from low melting point agarose gels and/or by electroelution from standard agarose gels. For -168aP2CAT, a 2.4kb fragment extending from SmaI to a PstI site was restricted from the plasmid, isolated and used for the injection. For -  
10 247aP2CAT, a 2.5kb fragment extending from NsiI to PstI was used for the injection.

Transgenes -1.7aP2CAT (PstI - PstI, 3.9kb) and -5.4aP2CAT (HindIII to NdeI -8.2kb) were similarly prepared for micro-injection.

15 The linear fragments were then used for microinjection into Swiss Webster (SW) mice one-cell zygotes and implanted into pseudopregnant foster females as described by Choi et al, J. of Virology, 61: 3013-3019 (October, 1987), the pertinent  
20 portions of which are incorporated by reference. In each instance, approximately 500 copies of the fragments in 5 picoliters were injected into each of about 300 eggs. SW mice were purchased from the NCI Frederick Animal Production Facility, Frederick,  
25 M.D.

Approximately 75% of the microinjected eggs were implanted into the foster females, with between 20-40% ultimately resulting in offspring.

Transgenes were identified, as described below, at a frequency of about 10-40% of the animals born.

**Identification Of Transgenic Animals With CAT-Containing Transgenes**

5           When the mice were weaned, total nucleic acids were extracted from a piece of the tail. To prepare genomic DNA from tissues, the tissue was homogenized in about 0.5ml 1x PBS and then incubated with Proteinase K (Boehringer Mannheim) at 50°C for 12-16  
10           hours. After digestion, cell debris was removed by centrifugation and the resulting supernatant containing the genomic DNA heated at 95°C for five minutes to inactivate the Proteinase K. Centrifugation at 15,000 rpm for 10 minutes followed  
15           and 15µl of the supernatant was used for PCR analysis. Positive transgenic animals with CAT-containing transgenes were identified by polymerase chain reaction, using primers that amplified a 280bp fragment of the SV40 small t antigen splice/poly-adenylation sequences. The two oligonucleotides  
20           used as primers were as follows:

5' <sub>4201</sub>GCACTCTATGCCTGTGTGG<sub>4220</sub>

and

3' <sub>4476</sub>TGAGGCTACTGCTGACACAC<sub>4441</sub>

25           wherein the subscript numbers refer to the SV40 genome.

          The polymerase chain reaction was conducted on a COY Temp Cycler (35 cycles of denaturation,

annealing and DNA synthesis) using standard techniques. Taq polymerase (ProMega Biotech, WI.) was used to catalyze the extension of annealed oligonucleotide primers. The animals that gave positive signals are identified in Table 1 below.

#### CAT Assays

CAT activity was measured in extracts prepared from the tissue of the offspring of the G<sub>1</sub> and subsequent generations. The mice were sacrificed at about 8 weeks of age, and samples of liver, spleen, thymus, kidney, brain, skeletal muscle, lung, heart and white adipose tissue obtained. Extracts from the mouse tissues were prepared for CAT analysis by manually homogenizing the various tissues in 0.3 - 0.5 ml of 0.25 M Tris-HCl (pH 8.0) and then sonicating the thus-prepared extracts in a water bath sonicator (Ultrasonics, Inc.) using 20 pulses of 50% power. After sonication, the extracts were centrifuged at 15,000 rpm for 10 minutes and the supernatant saved. The supernatant was heated at 65°C for 6 minutes in order to inactivate deacetylase enzymes present in the tissues and recentrifuged. The supernatant obtained following centrifugation was used for the CAT activity assays.

CAT assays were carried according to Lopata et al., (1984) the pertinent portions of which are incorporated by reference. For all of the

168aP2CAT, 247aP2CAT, and 1.7aP2CAT transgenic tissues and for the tissues from the 5.4aP2CAT transgenic animals expressing low levels of CAT enzyme, approximately 200-300 $\mu$ g of protein extract was assayed for 3 to 5 hours. In order to be within the linear range of the assay (<50% conversion of chloramphenicol to its acetylated forms), assays on extracts prepared from the fat, spleen and thymus of the 5.4aP2CAT mice were performed with 0.5 to 1 $\mu$ g of protein for 30 min. to 1 hr. The assays were performed in a total volume of 175 $\mu$ l containing 0.25 M Tris-HCl (pH 8.0), 0.46mM acetyl coenzyme A and 0.1m Ci of  $^{14}$ C-labeled chloramphenicol (E.I. du Pont de Nemours and Co., Inc.). The reactions proceeded at 37°C for 1-8 hours and were terminated by the addition of 1 ml of ethylacetate (J.T. Baker Chemical Co.). The samples were vortexed, centrifuged at 15,000 rpm for five minutes and the organic layer removed and dried under vacuum.

The dried material was resuspended in 25ml of ethyl acetate and spotted onto a thin layer chromatography (TLC) sheet (J.T. Baker Chemical Co.). The chromatography was performed for about thirty minutes using a solvent composed of 95:5 chloroform:methanol. The chromatography sheets were exposed to Kodak X-Ray film without an intensifying screen. The acetylated spots were cut out of the TLC and counted. Specific activities are presented

either as cpm/mg protein/min reaction time or cpm/ $\mu$ g protein/min reaction time, depending on the activity of the extract.

5       The approximate copy number was determined by quantitative dot blot analysis. Quantitative DNA dot blot analysis was carried out by hybridizing dot blots of genomic DNA to a  $^{32}$ P-labelled CAT probe and scintillation counting the hybridized spots.

10       Genomic DNA was prepared from mouse tissues by Proteinase K-SDS cell lysis followed by extraction with phenol/chloroform and ethanol precipitation. The ethanol precipitated DNA was resuspended in

15       Tris-HCl (10mM pH7.0-EDTA (1ml) and 1 $\mu$ g, 5 $\mu$ g and 10 $\mu$ g were used for dot blot analysis. Copy number was estimated by comparing the counts per minute (cpm) hybridized to a known amount of plasmid DNA.

The results of the foregoing experimentation are set forth in Table 1.

**TABLE 1****CAT Activity In The Tissues  
Of aP2CAT Transgenic Mice**

<u>Transgene<sup>b</sup></u>	<u>Copy #<sup>c</sup></u>	<u>Specific Activity In Tissues</u>						
		<u>Li</u>	<u>Sp</u>	<u>Th</u>	<u>Br</u>	<u>SM</u>	<u>Lu</u>	<u>Fa</u>
168aP2 14	50	0	33	17	1	ND	2	0
247aP2 2 <sub>1</sub>	675	12	5207	12,971	46	41	310	68
247aP2 2 <sub>2</sub>	5	7	33	17	1	ND	47	160
247aP2 3 <sub>1</sub> <sup>a</sup>	1255	12	1351	3,295	305	107	51	70
247aP2 3 <sub>1</sub> <sup>b</sup>	24	11	10	10	13	10	12	52
247aP2 3 <sub>2</sub>	21	0	9	12	24	ND	11	6
247aP2 5	45	ND	5	76	18	6	12	11
247aP2 10	15	0	33	55	7	2	57	20
247aP2 11	12	10	187	125	14	8	47	12
1.7aP2 3	5	0	456	241	ND	0	3	2
1.7aP2 6	45	4	814	402	6	3	7	34
1.7aP2 8	4	ND	484	1	425	0	19	8
5.4aP2 4 <sup>d</sup>	31	16	3800	730	12	120	>440 <sup>e</sup>	280
5.4aP2 17 <sup>d</sup>	5	1	2200	4400	4	ND	1400	>11,000 <sup>e</sup>
5.4aP2 18 <sup>d</sup>	5	8	450	360	29	43	73	860
5.4aP2 25 <sup>d</sup>	<5	27	93	130	0	ND	43	1300

Abbreviations: Li, liver; Sp, spleen; Th, thymus; Ki, kidney; Br, brain; SM, skeletal muscle; Lu, lung; He, heart; Fa, white adipose tissue; ND, not determined.

<sup>a</sup> CAT specific activities are presented as cpm acetylated chloramphenicol/mg protein/ min. reaction time. Zero specific activity represents specific activities of <0.5.

<sup>b</sup> 168aP2 contains the aP2 gene promoter from -168bp. 247aP2 begins at -247bp, while 1.7aP2 begins at -1.7kb and 5.4aP2 begins at -5.4kb. All of these constructs extend to +21 at the 3' end.

<sup>c</sup> Approximate copy number was determined by quantitative dot blot analysis.

<sup>d</sup> CAT specific activity x 10<sup>3</sup> for these animals.

<sup>e</sup> CAT activity was in the non-linear range of the assay, so that accurate specific activities could not be determined.

As shown in Table 1, in the independently-generated strains of transgenic mice containing either of the 168aP2CAT or 247aP2CAT constructs, little or no CAT activity was detected in adipose tissue. This lack of expression was independent of transgene copy number. Even the adipose tissue from animals containing 1200 copies contained very low levels of CAT enzymatic activity. Moreover, significant expression was seen in several non-adipose tissues, such as the spleen, thymus, lung and brain, although liver CAT levels were very low or undetectable (Table 1; Fig. 2A). Southern blot analysis of DNA from these animals revealed no gross rearrangement of the transgenes and in general, expression in all tissues was roughly proportional to copy number.

While the additional sequences in 1.7aP2CAT did not result in higher levels of CAT in adipose tissue, all four strains of transgenic animals containing the 5.4aP2CAT DNA expressed the transgene at very high levels in white and brown adipose tissue. See, Table 1. Three of the four transgenic strains (those that had  $\leq 5$  copies of the transgene had the highest levels of CAT activity in adipose tissue. None of the 5.4aP2CAT animals had appreciable activity in most other tissues, including liver as illustrated in Table 1. The addition of the 5' region from -1.7kb to -5.4kb not only increased the level of expression of the

transgene in adipose tissue relative to other tissues, but boosted the overall level of expression in 5.4aP2 CAT mice by about 1000-fold. (The specific activities for the 168-, 268- and 1.7aP2CAT animals are given in cpm/mg protein, while those for the 5.4aP2CAT animals are given in cpm/ $\mu$ g protein in Table 1).

#### RNA Isolation And Analysis

Transcription of CAT in tissues was determined by extracting RNA from the tissues and assaying the RNA in a RNase T1 protein assay. RNA was isolated from tissues according to the method of Chirgwin et al, (1979). RNase T1 protection assays were carried out as previously described (Melton et al, 1984); the temperature of hybridization of the probes to the RNA were 37°C for all probes containing CAT sequences, and 55°C for probes containing adipsin sequences. Integrity of the RNA was checked by Northern blot analysis and hybridization to the housekeeping gene, glyceraldehyde phosphate dehydrogenase (Fort et al, 1985). The probe used for the detection of CAT RNA was generated by cloning a HindIII to EcoRI fragment from 5.4aP2 CAT into the HindIII/EcoRI site of a Gemini vector (ProMega).

RNase protection analysis of RNA isolated from the tissues of these mice reflected the CAT activity



levels, indicating that the inclusion of the region from -1.7kb to -5.4kb resulted in increased mRNA levels derived from the transgene. Thus, sequences present in the aP2 gene between 1.7kb and 5.4kb upstream from the transcription start site enhanced expression dramatically in adipose tissue.

#### EXAMPLE 2

This example demonstrates that the enhancement of expression obtained with the -1.7kb to -5.4kb construct was independent of the attached marker gene, CAT.

The results described in Example 1 indicate that sequences within the 5' flanking region of the murine aP2 gene cause high levels of expression of linked genes in adipose tissue. To show that this enhancement of expression was independent of the linked marker gene and not the result of an interaction between the CAT/SV40 marker gene and aP2 sequences, another transgene, 5.4aP2adn was constructed. This construct contained the same -5.4kb to +21 of the aP2 gene linked to a hybrid cDNA/genomic adipsin gene and is illustrated in Figure 1C.

The construct was prepared by ligating a 500bp BamHI to BstEII (codon 167) cDNA fragment from the adipsin vector pMTpn-Adn (Rosen et al, Science, 244:1483-87 (1987) containing the AUG codon to a

4.8kb BstEII - EcoRI genome fragment containing the UGA stop codon and 4kb of 3' flanking sequences from lambda -ADN genomic clone. (Min and Spiegelman, Nucleic Acids Res., 14:8879-92, 1986). The aP2  
5 5.4kb to +21bp fragment (EcoRI - PstI) was then ligated upstream of the adipsin sequences.

The 5.4aP2ADN vector was then used to generate transgenic animals, in accordance with the experimental protocol described in Example 1. A  
10 10.1kb fragment (HindIII to PvuI) was used in the microinjection.

Seven strains of transgenic mice containing this transgene were produced and expression in various tissues was examined using an RNase T1  
15 protection assay. RNA was extracted from various tissues, including the spleen, brown fat and fat, in accordance with the method of Chirgwin, Biochemistry, 18: 5294-99 (1979) and RNAase T1  
20 protection assays were carried out as previously described (Melton et al, Nucleic Acid Res., 12: 7035-7056, 1984)). In this experiment, a 430bp fragment from the junction between the aP2 and adipsin sequences was cloned into the Bluescript (Stratagene, CA) vector and used to make a 475bp  
25 probe. The temperature of hybridization of the probes to the RNA were 55°C for all probes containing the adipsin sequences. The integrity of the RNA was checked by Northern blot analysis and

hybridization to the housekeeping gene,  
glyceraldehyde phosphate dehydrogenase (Fort et al,  
Nucleic Acid Res., 13: 1431-1442 (1985).

5 As shown in Figure 3, transcription from the  
transgene (detected as a 406nt band), and the  
endogenous adipsin gene (detected as bands of 352nt  
and 276nt), was found in both white and brown  
adipose tissue, while both were undetected in other  
tissues, such as spleen. This result was  
10 reproducible for all of the 5.4aP2adn transgenic  
mice examined.

These experiments show that the regulation of  
gene expression by the 5.4kb flanking region of the  
aP2 gene acts at the RNA level and that initiation  
15 occurs at the appropriate transcription start site.  
Moreover, results of the experimentation demonstrate  
that the aP2 promoter construct is clearly capable  
of enhancing the expression of different linked  
genes.

### 20 EXAMPLE 3

This example demonstrates that the aP2  
adipocyte specific enhancer element maps to the 5'  
distal end of the 5.4kb fragment isolated in Example  
1 and is the primary determinant of tissue-specific  
25 expression of this gene.

To more precisely map the enhancer element in  
the aP2 gene between -1.7kb and -5.4 kb, transient

transfections with a variety of constructs into undifferentiated and differentiated 3T3-F442A adipose cells were carried out. A basal promoter, -63aP2CAT (referred to as -64aP2CAT in Distel et al., 1987) was used, because this construct is inactive in both cell types and is deleted for the proximal regulatory elements (C/EBP, AP-1 and CCAAT box. (Distel et al, Cell, 49:835-844 (1987); Rauscher et al, Cell, 52:471-80 (1988)).

#### 10 Preparation Of Constructs

Plasmid -63aP2CAT, described by Distel et al, (Cell, 49:835-44 (1987)) includes a DNA sequence encoding CAT which is operatively associated with a basal promoter from murine aP2, comprising  
15 nucleotides -63 to +21. (-63aP2CAT is described as -64aP2CAT by Distel et al, and was obtained as a 5' deletion of the -168aP2CAT construct.) A series of restriction fragments from the 5' flanking region of the murine aP2 gene were inserted at the Hind III  
20 site of the -63aP2CAT vector, thereby placing the fragments upstream of the basal aP2 promoter. The restriction fragments inserted included a -5.4 to 1.7kb (EcoRI - EcoRI) restriction fragment, -5.4 to -3.8kb (EcoRI - Pvu 11) restriction fragment and a -  
25 5.4kb to -4.9 kb (EcoRI - XbaI) restriction fragment from the 5' flanking region of the aP2 gene. See, Figure 1B. Deletion constructs of the 5.4kb to

1.7kb fragments were made by blunt end ligation of the various restriction fragments into HindIII restricted and Klenow filled in -63aP2CAT. The origin and orientation of the DNA fragments inserted at the Hind III site of the basal -63aP2CAT vector are indicated in Figure 4. The thus-prepared aP2CAT fusion plasmids were then transferred into 3T3-F442A preadipocytes and adipocytes.

#### Cell Culture And Transfection

3T3-F442A preadipocytes and adipocytes were cultured as previously described. (Wilkison, et al, J. Biol. Chem., 265: 477-482 (1990) and Cook et al, Proc. Natl. Acad. Sci., 82: 6480-6484 (1985)). Transfections were done at day 2 after confluence in preadipocytes and days 4-6 after confluence in adipocytes. For transfections, 20µg of plasmid DNA was mixed with 5 ml of Dulbecco's medium containing 50mM Tris-HCl, pH 7.3 and either 100 µg/ml DEAE dextran for preadipocytes or 250 µg/ml DEAE dextran for adipocytes. The cells were incubated for 3 hours at 37°C, then the cells were shocked with 10% DMSO in PBS for 3 min. at 37°C. The monolayers were washed again with PBS, then incubated with serum-containing medium for 48 hours.

#### Assay For CAT Activity

CAT assays were done essentially by the method of Gorman et al, Mol. Cell Biol., 2: 1044-51 (1982) with minor modifications. First, the cell extracts  
5 were prepared by freezing and thawing cell pellets instead of sonicating. Second, the cell extracts were heated to 60°C for 7 min. to inactivate endogenous deacetylases. Third, the acetyl-CoA concentration in the assay was increased from 0.4 to  
10 2.4 mM to ensure linearity. The reactions were incubated at 37°C for 4 hours and stopped by the addition of 1 ml of ethyl acetate. Quantitation of CAT activity was done by cutting out spots from the silica gel containing the <sup>14</sup>C-labeled acetylated and  
15 unacetylated chloramphenicol and counting in scintillation fluid.

As shown in Figure 4, transfected plasmids containing from -5.4 kb at the 5' end to either -1.7kb, -3.8kb, or -4.9kb at the 3' end of the  
20 fragment (Fig. 1B) ligated upstream of -63bp in the aP2 promoter, stimulated higher levels of CAT expression in adipocytes, relative to the expression seen from the basal 63aP2CAT construct. The plasmid containing the -4.9kb to -3.8kb region ligated  
25 upstream of this same promoter/CAT sequences showed no enhancement of expression.

The EcoRI to XbaI restriction fragment mapping between -5.4kb and -4.9kb, when linked to the

minimal aP2 promoter, did not significantly enhance expression in preadipocytes, indicating that its enhancing function was differentiation-dependent.

#### Generation Of Transgenic Animals

5           To determine if the region between -4.9kb and -  
5.4kb also functioned as an enhancer in animals,  
transgenic mice containing this restriction fragment  
(linked upstream of the aP2 promoter at -63bp) were  
constructed and CAT activity in various tissues was  
10           measured.

          The -4.9 kb to -5.4 kb vector (termed  
512aP2CAT) was prepared for microinjection into  
mouse eggs by restricting the vector with AluI and  
NdeI.

15           A 4kb AluI-NdeI fragment was then separated  
from the bulk plasmid fragments on an agarose gel  
and used for injection into eggs. Swiss Webster  
one-cell zygotes ( $\approx 300$ ) were injected, as in Example  
1, with 5 picoliters containing about 500 copies of  
20           the fragment and implanted into pseudo-foster  
females as described. 56 animals developed from the  
eggs, and the animals were detected for the presence  
of the transgene, as described in Example 1. Tissue  
samples were analyzed for CAT activity, as  
25           previously described.

          Three of the animals expressed the transgene  
and, as shown in Table 2 below, the 512aP2CAT  
construct functioned in a fat-specific manner.

Adipose tissue contained from 3-50 fold higher levels of CAT activity than any other tissue in the animal, depending on the transgenic strain. This degree of adipocyte-specificity is generally consistent with the tissue-specific expression of the endogenous gene (Bernlohr et al, Biochem. Biophys. Res. Comm., 132: 850-855 (1985); Zezulak and Green, Mol. Cell Biol., 5: 419-421 (1985)). As was seen with the 5.4aP2CAT mice, transgene expression was orders of magnitude higher in the 512aP2CAT animals relative to the levels seen in transgenic animals lacking the enhancer sequence.

**TABLE 2****CAT Specific Activities in Tissues<sup>a</sup>**

<b>Transgene</b>	<b>Copy #<sup>b</sup></b>	<b>Li</b>	<b>Sp</b>	<b>Th</b>	<b>Br</b>	<b>SM</b>	<b>Lu</b>	<b>Fa</b>
512aP2 2 <sup>c</sup>	10	26	1	17	0	4.2	1	1235
512aP2 6	25	52	0	37	0	158	0	598
512aP2 11	5	25	166	195	57	178	0	9065

The 512aP2 construct contains from -5.4kb to -4.9kb in an inverted orientation ligated to the -63bp to +21bp fragment of the aP2 gene.

<sup>a,b,c</sup> See legend for Table 1.

**RNAase Protection Assay**

Since the 512aP2CAT molecule contained only a small region of the promoter, it was possible that the CAT activity that was detected in the adipose or other tissues of transgenic mice containing this construct was due to initiation at novel sites formed by the juxtaposition of sequences within the



512bp fragment and the promoter. RNAase T1 protection assays were therefore carried out using a probe that spanned the junction between the aP2 and CAT sequences. The results showed that RNA transcription initiated at the normal aP2 start site in the adipose tissue of strain 512aP2CAT 2 transgenic mice, resulting in protection of a fragment of 294nt. CAT transcripts were detected only in the adipose tissue of the 512aP2CAT mice, which reflects the lower levels of transgene expression in the other tissues.

#### Sequencing

The sequence of the 512 base pair aP2 adipose-specific enhancer element is set forth in SEQ ID NO: 1. The sequence was determined by dideoxy sequencing using the sequenase kit from USBIOCHEMICAL of Cleveland, Ohio. For sequencing -5.4 to -4.9, the EcoRI to XbaI fragment was inserted into pBst and M13 primers used for sequencing.

#### EXAMPLE 4

This example describes the preparation of deletion constructs of the 512aP2CAT transgene which retain the ability to deliver genetic material specifically to adipose tissue in culture.

Various restriction enzymes were used to cleave the 512 base pair aP2 enhancer sequence into three

smaller fragments containing DNA from -5.4 to -5.0kb, -5.4 to -5.2kb and -5.2 to -5.0kb. The restriction enzyme sites are indicated in Figure 1B. The Sst I site generated a 380 bp fragment (-5.4 to -5.0 kb), which was then cleaved in half with Sau3AI to generate the fragments from -5.4 to -5.2 kb and from -5.2 kb to -5.0 kb. These fragments were then ligated to the -63aP2CAT construct as previously described and transiently transfected by DEAE-dextran adsorption with 20 $\mu$ g of plasmid DNA. CAT activity was assayed as described in the previous Examples herein.

The results of this experiment showed that when linked to the -63aP2 construct, the -5.4 to -5.0 fragment had similar activity to the 512 bp fragment (-5.4 to -4.9 kb). When this fragment (-5.4 to -5.0 kb) was cleaved with Sau3AI, the 5' half (-5.4 to -5.2 retained enhancing activity while the 3' half had no detectable activity.

The three fragments can be used to generate transgenic mice in accordance with the procedure described herein, to determine whether the -5.4 to -5.0 and -5.4 to -5.2 kb fragments are sufficient to direct adipose-specific expression of a heterologous gene in vivo.

**EXAMPLE 5**

It has previously been shown that the first 1000 base pairs 5' of the transcription start site of the adipsin gene are sufficient to direct expression of genetic material in cultured adipocytes. This example describes the isolation of the promoter region from the murine adipsin gene which positively regulates expression in fat tissue in vivo.

**Preparation of ADN-CAT Fusion Genes**

The adipsin-CAT fusion plasmids were prepared using the pAE5 CAT vector (Spiegelman et al, J. Biol. Chem., 258: 10083-10089 (1983)) and adipsin sequences from lambda-ADN (Min and Spiegelman, Nucleic Acids Res., 14: 8879-8892 (1986)). The pAE5 vector was derived from the plasmid pSV1xCAT-AB3 (Celander et al, Nature, 312, 159-162 (1984)) and contains a mutant SV40 early promoter region between XhoI and HindIII sites of the CAT gene and the Akv viral enhancer (-443 and -110 of the Akv lung terminal repeat, Van Beveren et al, J. Virol., 41: 542 (1983)) 3' of the CAT gene at the BglII site of pSV1xCAT-AB3.

The pADN (-950) vector was prepared by excising the XhoI-HindIII SV40 promoter fragment of pAE5 and replacing it with a BamHI-RsaI fragment spanning 0.95 kilobases of the 5' flanking downstream to 35bp 3' of the transcription start site of the adipsin

gene. A similar plasmid containing an XbaI-RsaI fragment of approximately 700bp of the 5' flanking sequence (pADN-CAT (-700)) was also constructed. Subsequent 5' deletions of the adipsin upstream region were made from (pADN-CAT (-700)) by linearizing it with XhoI and then digesting for various times with Bal 31 nuclease. The deleted ends were repaired with T4 polymerase and XhoI linkers attached. The 5' deleted fragments were excised with HindIII and then ligated into the pAE5 vector minus the XhoI-HindIII promoter fragment. The 5' ends of all deletions were identified by DNA sequencing of plasmids. The following deletion plasmids were prepared in accordance with the foregoing: pADN-CAT (-344); pADN-CAT (-305); pADN-CAT (-284); pADN-CAT (-250); pADN-CAT (-215); pADN-CAT (-114); and pADN-CAT (-38).

#### Cell Culture and Transfections

The thus-prepared plasmids were then transfected into 3T3-F442A preadipocytes and adipocytes to delineate the regions within the adipsin promoter which direct differentiation dependent gene expression in vivo. Cell cultures and transfections were carried out as described in Example 3, except that transfections were conducted with 10 $\mu$ g of each plasmid.

### Assays for CAT Activity

CAT assays were done essentially by the method of Gorman et al, Mol. Cell Biol., 2: 1044-1051 (1982) with some modifications. First, the cell extracts were prepared by freezing and thawing cell pellets instead of sonicating. Second, the cell extracts were heated to 60°C for 7 min. to inactivate endogenous deacetylases (Mercola et al, Science, 227: 266-70 (1985)). Third, the acetyl-CoA concentration in the assay was increased from 0.4 to 2.4 mM to ensure linearity. The reactions were incubated at 37°C for 4 hours and stopped by the addition of 1 ml of ethyl acetate. Quantitation of CAT activity was done by cutting out spots from the silica gel containing the <sup>14</sup>C-labeled acetylated and unacetylated chloramphenicol and counting in scintillation fluid.

The results of the CAT assays of cell extracts of the pADA-CAT (-950) transfection and variations are set forth in Figure 6. As illustrated in Figure 6, upon transfection with pADN-CAT (-950), preadipocyte extracts exhibited only background CAT activity, whereas adipocyte extracts showed significant CAT activity above background. No CAT activity was observed in mock transfection lacking plasmid DNA or in transfections with pADN-CAT (-950)F in which the 985-bp adipsin 5'-flanking region was in an inverted (3' to 5') orientation

upstream of the CAT gene. A positive control used for transfection efficiency, the original PAE5 vector, exhibited abundant CAT expression in both preadipocytes and adipocytes. This plasmid contains SV40 promoter sequences upstream of the CAT coding region and the Akv enhancer downstream of the CAT gene. This vector also demonstrated that the Akv enhancer in the pADN-CAT construction does not exhibit inherent differentiation-dependent activity since CAT activity in adipocytes transfected with the PAE5 vector was similar to that observed in preadipocytes. Thus, these experiments strongly suggest that the adipsin gene contains sufficient regulatory information within 950bp of the 5'-flanking sequence to direct differentiation-dependent expression of a heterologous gene in fat cells.

The transfections with the 5' deletions of pADN-CAT allowed a more precise delineation of the regions within the adipsin promoter responsible for directing differentiation-dependent expression. The CAT activity of the pADN-CAT (-700) deletions are set forth in Table 3 below.

**TABLE 3**

<u>CAT Activity</u>			
	<u>Plasmid</u>	<u>Preadipocyte</u> <u>% conversion to acetylated forms</u>	<u>Adipocyte</u>
5	pAU3CAT	7.0 (1.00)	12.9 (1.00)
	pADN-CAT (-344)	<0.1 (0.01)	5.6 (0.43)
	pADN-CAT (-305)	<0.1 (<0.01)	10.1 (0.79)
	pADN-CAT (-284)	<0.1 (0.01)	3.9 (0.33)
	pADN-CAT (-250)	<0.1 (0.01)	5.0 (0.39)
10	pADN-CAT (-215)	<0.1 (0.01)	8.5 (0.66)
	pADN-CAT (-114)	<0.1 (0.01)	9.8 (0.76)
	pADN-CAT (-38)	2.3 (0.32)	0.8 (0.06)

15 In Table 3, the base number refers to the 5' boundary of adipsin sequences, as determined by sequencing. Preadipocytes and adipocytes were transfected with DEAE-dextran and 2 $\mu$ g of pAU3CAT, a positive control vector (Celander et al, Nature, 312: 159-162, (1984)), or 10 $\mu$ g of other plasmids.

20 CAT activity was determined as described. Background counts from mock transfections were subtracted from all data. The numbers in the parentheses present data normalized to pAU3CAT, which is given as 1.00. The increase in CAT

25 activity in preadipocytes and the decrease in

adipocytes between the -114 and -38 deletions were observed in at least 15 separate transfections with six different plasmid DNA preparations and were also observed in pooled stable transfectants.

5           Deletions from -950 to -344 caused no significant change in CAT activity in extracts of preadipocytes or fat cells (not shown). The data in Table 3 illustrate that further deletions from -344 toward the transcription start site caused only  
10           small changes in CAT expression until the deletions reached the region from -114 to -38 (Table 3). In preadipocytes, CAT activity markedly increased (at least 20-fold) above the background level when the  
15           region from -114 to -38 was deleted, suggesting that one or more negative regulatory elements had been lost. In adipocytes, deletion to -38 caused a dramatic (>9-fold) drop in CAT expression, suggesting that there was an element between  
20           positions -114 and -38 which positively regulated expression from the adipsin promoter in these cells. The rise in CAT activity in preadipocytes and the drop in activity in fat cells which occurred upon deletion from -114 to -38 were both highly reproducible.

#### 25           Generation of Transgenic Animals

          The foregoing experiment demonstrated that constructions comprising a -114 to +35 segment of the adipsin promoter are preferentially expressed in



adipose cells in culture. In order to determine whether the same promoter sequence is capable of directing the expression of a functional gene in vivo, transgenes containing 114 base pairs of the adipsin 5' flanking region were tested in mice. The vector was prepared for microinjection into mouse eggs by restricting the vector with NdeI and PvuI. A 3kb NdeI-PvuI fragment was then separated from the bulk plasmid fragments on an agarose gel and used for injection into eggs. Swiss Webster one-cell zygotes were injected as in Example 3.

Two strains of transgenic mice were generated (114ADN CAT 3 and 114ADN CAT 13). Two male offspring were sacrificed at 1-2 months of age and CAT assays conducted on tissue extracts, as previously described. The specific activities reported in Table 4 below represent the average of the two animals from each strain.

TABLE 4

20	<u>Tissue</u>	<u>Specific Activity (cpm/mg/min)</u>	
		114adnCAT 3	114adnCAT 13
	Liver	0.36	0
	Salivary Gland	0.4	0
	Spleen	1.1	1.2
25	Kidney	0	not done
	Thymus	2.0	8.5
	Brown Fat	12.4	not done
	White Fat	38.1	11.6

The results demonstrate that the sequence comprising -114 to +35 of the murine adipsin gene is sufficient to direct the fat specific expression of a heterologous gene in vivo.

5           The sequence of the 149bp adipsin promoter, which has previously been published in connection with the cloning of the adipsin gene, is set forth in SEQ ID NO: 2. Larger sequences, which contain  
10           this minimal promoter together with additional DNA from the 5' flanking region of the adipsin gene, will also be useful in accordance with the present invention.

#### EXAMPLE 6

15           This Example describes the construction of a transgenic promoter vector (TAP), including the 512bp enhancer sequence and minimal aP2 promoter, which is useful in generating transgenic animals in accordance with the present invention.

20           -4.9kb           -5.4kb/-63bp   +21  
Cloning The Promoter 5' XbaI....EcoRI/HindIII...PstI

25           The -5.4kb to -4.9 kb EcoRI to XbaI fragment of the murine aP2 gene was subcloned into pBS, to yield plasmid 512pBS. 512pBS was then cut with EcoRI, filled in with Klenow, then cut with XbaI and the 512 bp insert prepared for cloning.

            -63aP2CAT, described in Example 1, was cut with HindIII, filled in with Klenow, then cut with PstI

and an 84bp fragment prepared.

The commercially available vector, bluescript SKII+ (Stratagene, La Jolla, CA), was cut with XbaI and PstI and the appropriate fragment isolated.

5       The DNA from each of the foregoing steps was then ligated in accordance with conventional techniques.

#### Cloning The Terminator

10       The 800bp SV40 small t intron and polyadenylation site from the plasmid pET<sub>GL</sub> was removed from the vector by BglII and BamHI digestion. The 800 bp fragment was then ligated into BamHI cut pBS+ and a clone in the proper orientation was isolated. (pET<sub>GL</sub> was generated in  
15       our laboratory; however vectors containing the SV40 small t intron and polyadenylation signal are readily available to persons skilled in the art and can alternatively be employed).

#### Final Construction of TAP

20       The terminator clone was cleaved with KpnI and XbaI and the 800bp fragment isolated. The promoter clone was cleaved with KpnI and XbaI and the 3.6kb vector isolated and prepared for cloning. The thus prepared fragments were then ligated.

25       A schematic map of the TAP vector is illustrated in Figure 7.

#### EXAMPLE 7

This example describes the procedure for preparing a recombinant DNA sequence, TAP-MYOD, in accordance with the present invention and also describes how to use the recombinant sequence in the generation of transgenic mice which are leaner than nontransgenic controls which lack the transgene.

#### Preparation Of Constructs

Mouse MYOD cDNA is isolated as a .9 kb HindIII to EcoRI restriction fragment from a vector available from Harold Weintraub, Fred Hutchinson Cancer Center, Seattle, Washington. The cDNA sequence for the mouse MYOD gene was presented in Davis et al, Cell, 51:987-1000 (1987). This fragment contains the entire MYOD coding sequence. The TAP aP2 promoter vector obtained in accordance with Example 6 is then cleaved with EcoRI and HindIII and the fragment containing MYOD is ligated into the digested TAP vector, to yield a plasmid which contains the aP2 enhancer/basal promoter upstream of the cDNA sequence for MYOD (TAP-MYOD). A schematic map of TAP-MYOD is provided in Figure 8.

#### Generation Of Transgenic Mice

The TAP-MYOD construct is prepared for microinjection by Not I and Bam HI digestion. A 2.5 kb fragment containing the recombinant aP2MYOD

sequence is then isolated and used for microinjection. About 125 eggs are microinjected and replaced into pseudopregnant mice.

5 Tail blots of the pups are performed and probed with radioactively labeled MYOD cDNA probes, thereby identifying the pups which contain the MYOD sequences. The transgenes are detected by Southern blotting of HindIII and EcoRI digested DNA, in accordance with the procedure described by Hogan et al, Manipulating the Mouse Embryo, Cold Spring  
10 Harbor Press (1988). RNA is isolated from fat pads and subjected to Northern blot analysis to test for expression of the MYOD sequences in adipose tissue.

15 The pups are maintained on a diet of standard laboratory feed after weaning and growth and weight charts are kept on the transgenic animals and age and sex matched non-transgenic controls.

The G0 progeny are mated at six weeks of age to produce a G1 generation. The G1 generation progeny  
20 are tested for the presence of the MYOD transgene, as described above and growth and weight charts maintained. Transgenic animals of the G1 generation which express the transgene are sacrificed at about two to three months of age and fat pads, including  
25 gonadal, peri-adrenal, subcutaneous and abdominal are removed, weighed and measured to determine the effect of expression of the transgene in adipose tissue.

The mice that carry the MYOD transgene are leaner than the age and sex matched non-transgenic controls.

#### EXAMPLE 8

5           This example describes the procedure for  
preparing a recombinant DNA sequence, TAP- $\alpha$ 2-  
adrenergic, in accordance with the present  
invention, and also describes how to use the  
10 recombinant sequence in the generation of transgenic  
mice.

#### Preparation Of Constructs

          The  $\alpha$ 2-adrenergic receptor DNA is isolated as a  
1.5 kb NcoI and HindIII restriction of the human  
genomic clone. The  $\alpha$ 2 adrenergic receptor DNA is  
15 derived from the clone described by Kobilka et al,  
Science, 238:650 (1987) and is available from Robert  
Lefkowitz, Duke University Medical School, (Durham,  
N.C.). The 1.5 bp  $\alpha$ 2 adrenergic restriction  
fragment which is isolated contains the entire  
20 intronless gene encoding  $\alpha$ 2 adrenergic. An adaptor  
oligonucleotide is used to recreate some of the non-  
coding leader sequence of the  $\alpha$ 2 adrenergic receptor  
mRNA and also to join the NcoI site into the PstI  
site of TAP. The oligomers used are as follows:

25           5' CAT GGG CGC AAA GCT GCC CTG CA 3'  
          5' GGG CAG CTT TCG GCC 3'

The TAP vector, obtained in accordance with Example 7, is then cleaved with PstI and HindIII and the vector isolated. To construct the final vector, the 1.5 kb NcoI and HindIII restriction fragment, oligomers and digested TAP are ligated together to obtain the TAP- $\alpha$ 2 adrenergic construct.

A schematic map of the TAP- $\alpha$ 2 adrenergic construct is provided in Figure 9.

#### Generation Of Transgenic Mice

The TAP- $\alpha$ 2 adrenergic receptor construct is prepared for microinjection by digesting with NotI and BamHI and isolating a 3.0 kb fragment. Two hundred ninety-seven eggs are implanted into pseudopregnant mice as described.

The eighty pups that are born are tested for the presence of the  $\alpha$ 2 adrenergic transgene by conducting an analysis of genomic DNA. Genomic DNA is prepared and the DNA restricted with XbaI, run on 0.8% agarose gels, Southern blotted and probed with  $\alpha$ 2 adrenergic radioactively labeled cDNA, thereby identifying the pups which contain the  $\alpha$ 2 adrenergic sequences. A unique 2.1 kb band is detected in six of the animals. RNA is isolated from fat pads and subjected to Northern blot analysis to test for expression of the  $\alpha$ 2 adrenergic sequences in adipose tissue.

Pups are maintained on a diet of standard laboratory feed after weaning and growth and weight

charts are kept on the transgenic animals and age and sex matched non-transgenic controls.

5       The G0 progeny are mated at six weeks of age to produce a G1 generation. The G1 generation progeny are tested for the presence of the  $\alpha 2$  adrenergic sequence, as described above, and growth and weight charts maintained. Transgenic animals of the G1 generation which express the transgene are sacrificed at about two to three months of age and  
10       fat pads, including gonadal, peri-adrenal, subcutaneous and abdominal are removed, weighed and measured to determine the effect of expression of the transgene in adipose tissue.

15       The mice that carry the  $\alpha 2$  adrenergic transgene are leaner than the age and sex-matched non-transgenic controls.

#### EXAMPLE 9

20       This example describes the experimental protocol for generating a leaner strain of feed animal in accordance with the present invention.

      In this example, the linearized 2.5 kb fragment containing the 512aP2-MYOD transgene obtained in accordance with Example 7 was microinjected into zygotes obtained from fertilized pigs.

25       The procedures for collecting and transferring of zygotes has been described (Hammer et al., Nature, 315: 680-683 (1985); Wall et al., Biol. of



Reprod., 32: 645-651 (1985). Briefly, donor gilts will be fed 15 mg altrenogest (17 $\alpha$ -allyl-estratriene-4-9-11.17 $\beta$ -ol-3-one; RU-2267; Roussel Uclaf Company, Paris) daily for about 5-9 days beginning on days 12-16 of the oestrous cycle. Gilts will be injected with 1500-2000 IU pregnant mare serum gonadotrophin 24-30 h after the last feeding of altrenogest and with 500 IU human chorionic gonadotrophin (hCG) after an additional 78 h. Gilts are then mated or artificially inseminated 18-36 h after the hCG injection.

Ova are surgically collected from the gilts 57-60 h after the hCG injection by flushing culture medium from the uterotubal junction through the cannulated infundibular end of each oviduct. Ova are collected and maintained in Brinster's medium for ovum culture (BMOC-3; Brinster, 1972) containing 1-5% (w/v) bovine serum albumin (ICN Immuno Biologicals, Lisle, IL. U.S.A.).

Ova are centrifuged several minutes to stratify the cytoplasm and permit visualization of pronuclei and nuclei by interference-contrast microscopy (Wall et al., 1985). After centrifugation, ova are held for microinjection by a blunt holding pipette (50  $\mu$ m diameter), while the tip of an injection pipette (1-5  $\mu$ m diameter) is inserted into one pronucleus of zygotes or each nucleus of two-cell ova. The nuclear structures are injected with about 300 to

500 copies of linear fragment containing the 512aP2-MYOD transgene. After injection, the ova are transferred to recipient gilts by aspirating them into sterile polyethylene tubing, inserting the tubing through the infundibulum and expelling the ova into the lower ampulla.

The offspring will be allowed to develop from these eggs.

At about 4 weeks of age, total nucleic acids are extracted from a piece of the tail of the offspring and used for DNA blot hybridization to determine which of the piglets carry the 512aP2-MYOD transgene. Again, using a radioactively-labelled MYOD cDNA probe, the animals which carry the 512aP2-MYOD transgene are detected by their ability to generate hybridization signals above background. DNA can be further analyzed using an appropriate restriction enzyme(s) and Southern blotting.

The piglets are weaned at about 4 to 6 weeks of age and then housed either separately or in small groups. After weaning, the piglets are maintained on a controlled diet of solid food and water and their growth, weight, and carcass fat composition measured periodically. Non-transgenic age and sex matched piglets serve as controls. Carcass fat thickness is measured on the live animals using real time ultrasound. Ultrasound scanning measurements on the piglets are obtained from a variety of

locations in accordance with the conventional wisdom in the art. See, Terry et al, J. Anim. Sci., 67:1279-1284 (1989).

5       The pigs carrying the transgene exhibit less carcass fat and are leaner than the controls. Maximal expression of the MYOD transgene is in adipose tissue.

10       From the above description, it is apparent that the objects of the present invention can be readily achieved. While only certain embodiments have been set forth, alternative embodiments and various modifications will be apparent from the above descriptions to those skilled in the art. For example, other adipose specific control sequences  
15       may be obtained from other adipose differentiation-linked genes and/or other structural genes exhibiting an effect on adipose tissue metabolism may be employed. These and other alternatives are considered equivalents and within the spirit and  
20       scope of the present invention.

SEQUENCE LISTING

## (1) GENERAL INFORMATION:

- (i) APPLICANT: SPIEGELMAN, BRUCE M.  
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- (ii) TITLE OF INVENTION: Adipocyte-Specific DNA Sequences And Use Thereof In The Production Of Transgenic Animals Exhibiting Altered Fat Metabolism.

- (iii) NUMBER OF SEQUENCES: 2

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## (v) COMPUTER READABLE FORM:

- (A) MEDIUM TYPE: Diskette, 3.50 inch, 720Kb storage  
(B) COMPUTER: IBM Personal System 2; Model 30  
(C) OPERATING SYSTEM: MS/DOS  
(D) SOFTWARE: WordPerfect 5.0

## (vi) CURRENT APPLICATION DATA:

- (A) APPLICATION NUMBER:  
(B) FILING DATE:  
(C) CLASSIFICATION:

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- (A) APPLICATION NUMBER: US 07/589,971
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## (2) INFORMATION FOR SEQUENCE ID NO:1:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 512 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: genomic DNA

## (iii) HYPOTHETICAL: no

## (iv) ANTI-SENSE: no

## (vi) ORIGINAL SOURCE:

- (A) ORGANISM: Mus musculus
- (B) STRAIN: Swiss Webster
- (D) DEVELOPMENTAL STATE: embryonic
- (F) TISSUE TYPE: embryonic fibroblast
- (G) CELL TYPE: fibroblast
- (H) CELL LINE: 3T3-F442A

(vii) IMMEDIATE SOURCE:

(A) LIBRARY: genomic  
(B) CLONE: aP2911

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

CCAGCAGGAA TCAGGTAGCT GGAGAATCGC ACAGAGCCAT	40
GCGATTCTTG GCAAGCCATG CGACAAAGGC AGAAATGCAC	80
ATTTACCCCA GAGAGAAGGG ATTGTAGTCA GCAGGAAGTC	120
ACCACCCAGA GAGCAAATGG AGTTCCCAGA TGCCTGACAT	160
TTGCCTTCTT ACTGGATCAG AGTTCACTAG TGGAAGTGTC	200
ACAGCCCAAA CACTCCCCCA AAGCTCAGCC CTCCTTGCC	240
TTGTAACAAT CAAGCCGCTC CTGGATGAAC TGCTCCGCCC	280
TCTGTCTCTT TGGCAGGGTT GGAGCCCACT GTGGCCTGAG	320
CGACTTCTAT GGCTCCCTTT TCTGTGATTT TCATGGTTTC	360
TGAGCTCTTT TCCCCGCTT TATGATTTTC TCTTTTTGTC	400
TCTCTCTTGC TAAACCTCCT TCGTATATAT GCCCTCTCAG	440
GTTTCATTTT TGAATCATCT ACTGTGAACT ATTCCCATTG	480
TTTGCCAGAA GCCCCCTGGT TCTTCCTTCT AG	512

## (3) INFORMATION FOR SEQUENCE ID NO:2:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 149 nucleotides
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: genomic DNA

## (iii) HYPOTHETICAL: no

## (vi) ORIGINAL SOURCE:

- (A) ORGANISM: Mus musculus
- (B) STRAIN: Swiss Webster
- (D) DEVELOPMENTAL STAGE: embryonic
- (F) TISSUE TYPE: embryonic fibroblast
- (G) CELL TYPE: fibroblast
- (H) CELL LINE: 3T3-F442A

## (vii) IMMEDIATE SOURCE:

- (A) LIBRARY: genomic
- (B) CLONE: lambda adipsin

## (x) PUBLICATION INFORMATION:

- (A) AUTHORS: HUNT, CLAYTON R.  
RO, JASON H.S.  
DOBSON, DEBORAH E.  
MIN, HYE YEONG  
SPIEGELMAN, BRUCE M.
- (B) TITLE: Adipocyte P2 Gene: Developmental expression and homology of 5' flanking sequences among fat cell-specific genes.
- (C) JOURNAL: Proc. Natl. Acad. Sci.
- (D) VOLUME: 83
- (F) PAGES: 3786-3790
- (G) DATE: 00-JUN-1986



(K) RELEVANT RESIDUES IN SEQ ID NO:2:  
FROM 1 to 149

(X) PUBLICATION INFORMATION:

(A) AUTHORS: MIN, HYE YEONG  
SPIEGELMAN, BRUCE M.

(B) TITLE: Adipsin, the adipocyte serine  
protease

(C) JOURNAL: Nucleic Acids Research

(D) VOLUME: 14

(E) NUMBER: 22

(F) PAGES: 8879-8892

(G) DATE: 1986

(K) RELEVANT RESIDUES IN SEQUENCE ID NO:2:  
From 1 to 149

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

GGGCTGACAC ACCGAAAGTG CAGACTCCCC TTCCCTAGTT	40
GGTTTTCTGC CCACCAGGCA AGGGGCAGGA GGTAAGAGGC	80
AGGAGTCCAT AAAACAGCCC TGAGAGCCTG CTGGGTCAGT	120
GCCTGCTGTC AGA ATG CAC AGC TCC GTG T	149

Having described the invention, what is claimed is:

1. A DNA sequence comprising an adipose specific DNA sequence operatively linked to a DNA coding sequence coding for a recombinant protein having a either a lipolytic or lipogenic effect on adipose tissue.  
5
2. A DNA sequence according to claim 1, wherein the adipose-specific DNA sequence comprises an enhancer sequence operatively linked to said DNA coding sequence through a functional promoter.
3. A DNA sequence according to claim 2, wherein the enhancer sequence is an aP2 enhancer.
4. A DNA sequence according to claim 3, wherein the aP2 enhancer includes has the nucleotide sequence set forth in SEQ ID NO:1 or fragments, variants or deletions thereof which retain the ability to direct expression of genetic material specifically in adipose tissue in vivo.  
5
5. A DNA sequence according to claim 1, wherein the adipose-specific DNA sequence comprises a promoter sequence.

6. A DNA sequence according to claim 5, wherein the promoter sequence is isolated from the adipsin promoter.

7. A DNA sequence according to claim 6, wherein the adipsin promoter includes the nucleotide sequence set forth in SEQ ID NO:2.

8. A method for producing animals exhibiting altered fat tissue metabolism transgenically comprising the steps of introducing a DNA sequence comprising an adipose specific DNA sequence operatively linked to a DNA coding sequence coding for a recombinant protein having a either a lipolytic or lipogenic effect on adipose tissue into the germ line of an animal or ancestor thereof at an embryonic stage and selecting from the transgenic offspring which develop the animals which exhibit a reduction or increase in fat tissue content compared to non-transgenic animals of the same species.

9. A method according to claim 8, wherein the adipose-specific DNA sequence comprises an enhancer sequence operatively linked to said DNA coding sequence through a functional promoter.

10. A method according to claim 9, wherein the enhancer sequence is an aP2 enhancer.

11. A method according to claim 10, wherein the DNA coding sequence codes for a recombinant protein which has a lipolytic effect on fat tissue and the transgenic animals are leaner than the non-transgenic controls.

12. A method according to claim 10, wherein the DNA coding sequence codes for a recombinant protein which has a lipogenic effect on fat tissue and the transgenic animals are more obese than the non-transgenic controls.

13. A method according to claim 10, wherein the aP2 enhancer includes the nucleotide sequence set forth in SEQ ID NO:1 or fragments, variants or deletions thereof which retain the ability to direct expression of genetic material specifically in adipose tissue in vivo.

14. A method according to claim 8, wherein the adipose-specific DNA sequence comprises a promoter sequence.

15. A method according to claim 14, wherein the promoter sequence is isolated from the adipsin promoter.

16. A method according to claim 15, wherein the adipsin promoter includes the nucleotide sequence set forth in SEQ ID NO:2.

17. A transgenic animal, excluding humans, whose germ line and somatic cells contain a DNA sequence comprising an adipose specific DNA sequence operatively linked to a DNA coding sequence coding  
5 for a recombinant protein having a lipolytic or lipogenic effect on adipose tissue.

18. A transgenic animal according to claim 17, wherein the adipose-specific DNA sequence comprises an enhancer sequence operatively linked to said DNA coding sequence through a functional promoter.

19. A transgenic animal according to claim 18, wherein the enhancer sequence is an aP2 enhancer.

20. A transgenic animal according to claim 19, wherein the aP2 enhancer includes the nucleotide sequence set forth in SEQ ID NO:1 or fragments, variants or deletions thereof which retain the  
5 ability to direct expression of genetic material specifically in adipose tissue in vivo.

21. A transgenic animal according to claim 20, wherein the recombinant protein has a lipolytic effect on adipose tissue and the animals are leaner

than non-transgenic controls which do not bear the transgene.

22. A transgenic animal according to claim 20, wherein the recombinant protein has a lipogenic effect on adipose tissue and the animals are more obese than non-transgenic controls which do not bear the transgene.

5

23. A transgenic animal according to claim 17, wherein the adipose-specific DNA sequence comprises a promoter sequence.

24. A transgenic animal according to claim 23, wherein the promoter sequence is isolated from the adipsin promoter.

25. A transgenic animal according to claim 24, wherein the adipsin promoter includes the nucleotide sequence set forth in SEQ ID NO:2.

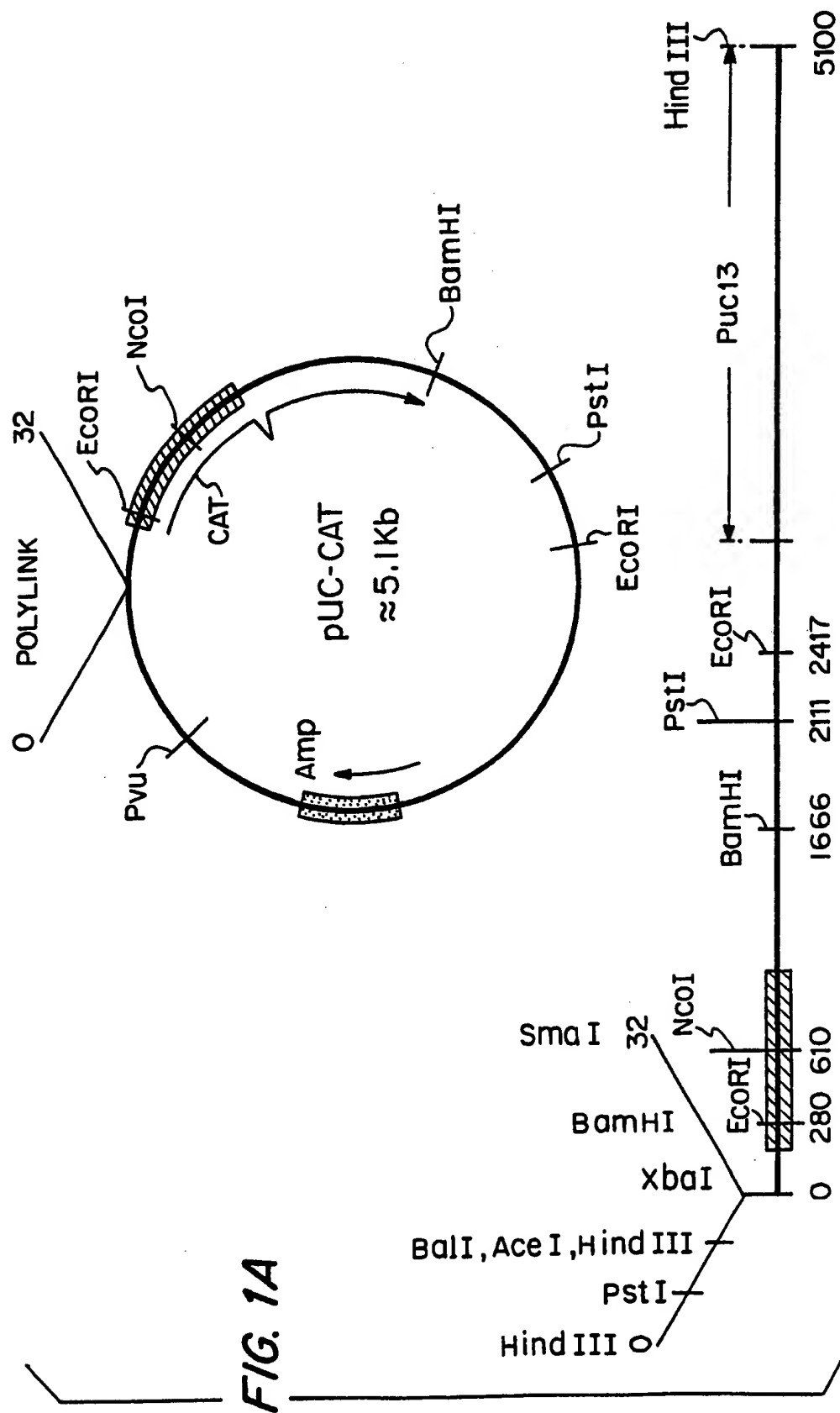
26. An adipose-specific enhancer element isolated from the 5' flanking region of the murine aP2 lipid binding protein gene expressed primarily in differentiated adipocytes, said DNA sequence, when operatively linked to a DNA sequence coding for a recombinant protein, is capable of directing the expression of the protein specifically in adipose tissue in vivo.

5

27. A DNA sequence according to claim 26,  
wherein the enhancer has the nucleotide sequence set  
forth in SEQ ID NO:1 or fragments, variants or  
deletions thereof which retain the ability to direct  
the expression of a DNA sequence encoding a  
recombinant protein in viyo.



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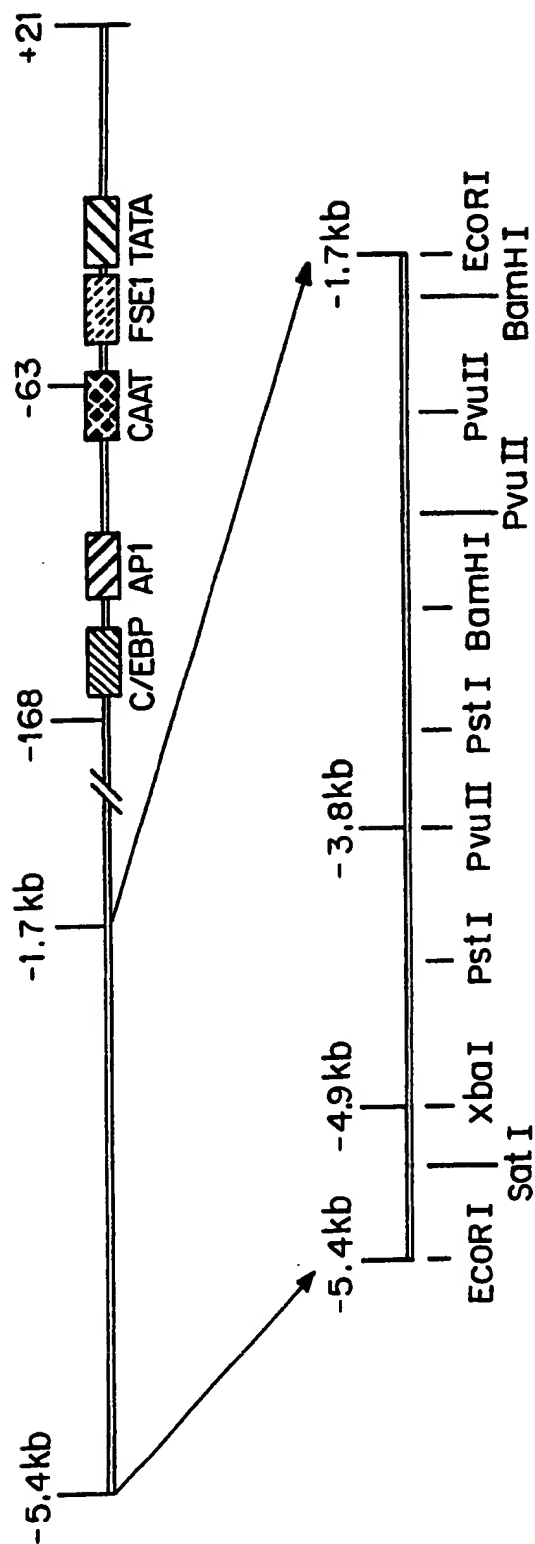
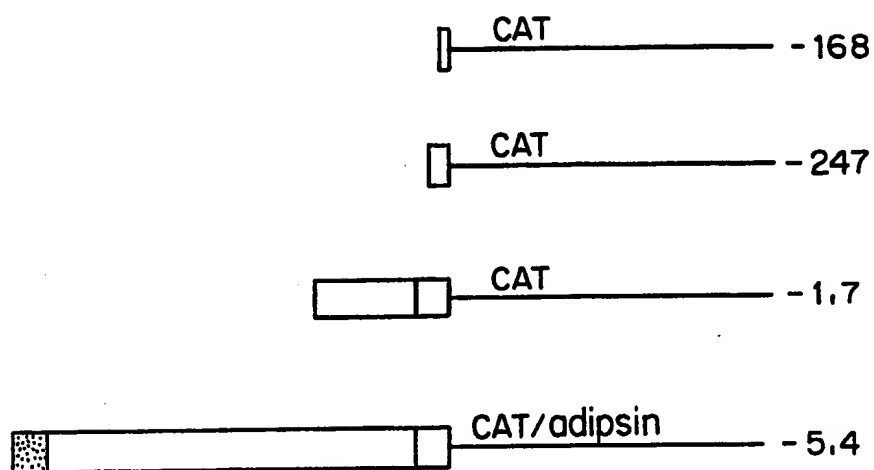


FIG. 1B

SUBSTITUTE SHEET

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*FIG. 1C*

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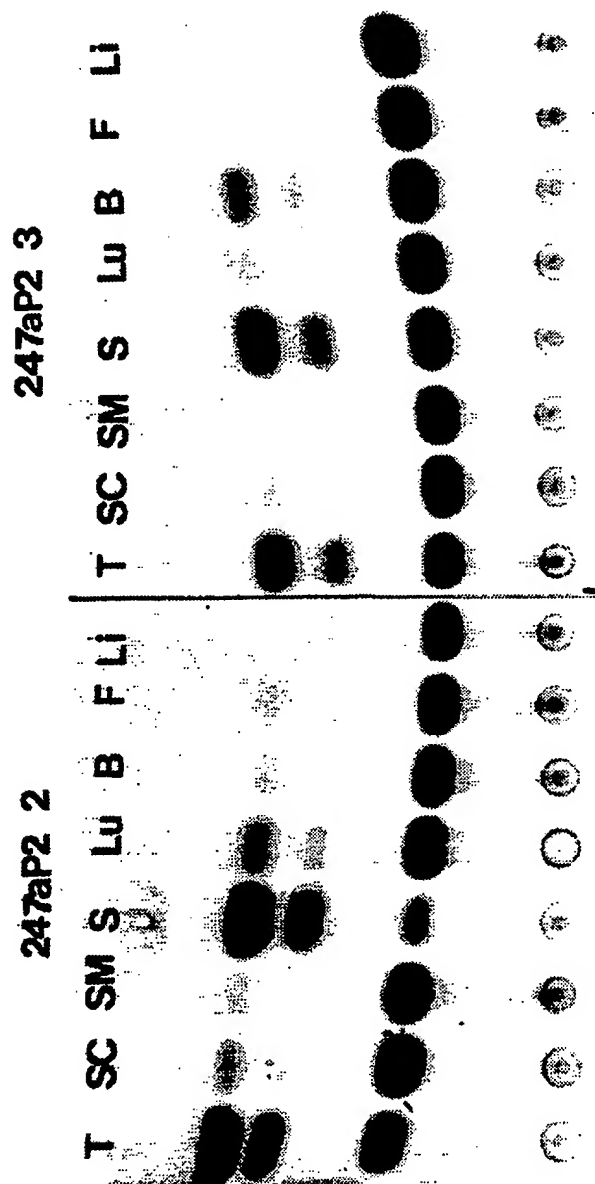


FIG. 2A

SUBSTITUTE SHEET

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B BF H Li Lu SG Sp Th F

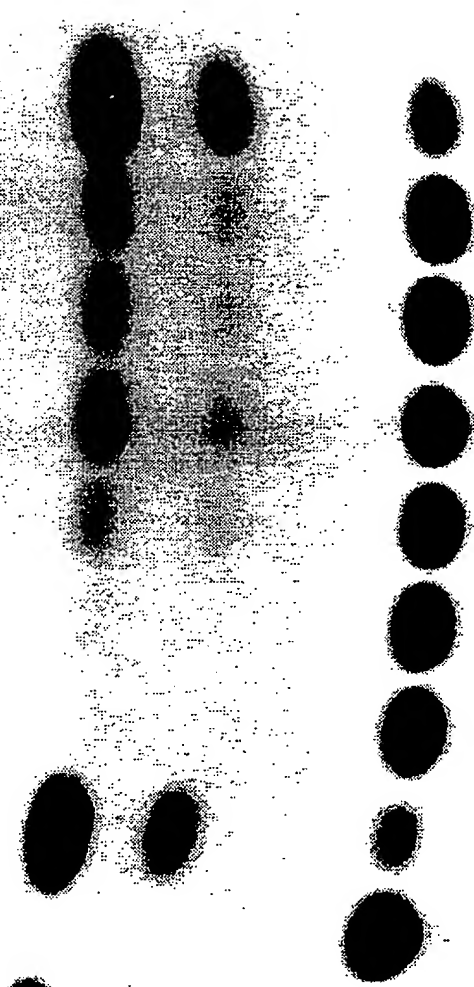


FIG. 2B

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N T N T N T  
S S BF BF F F

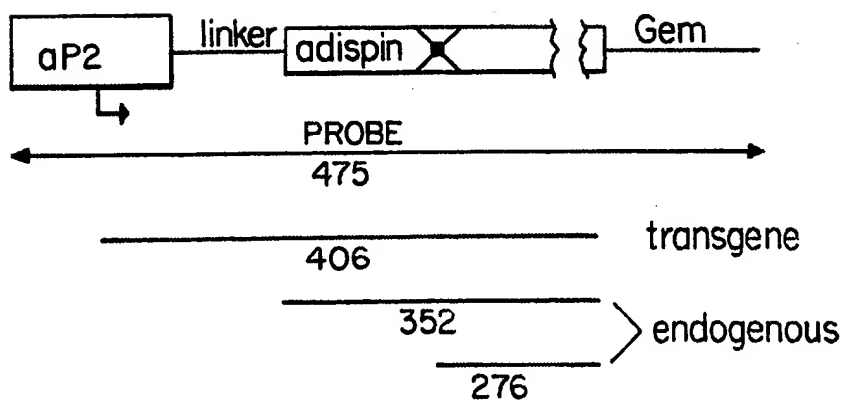
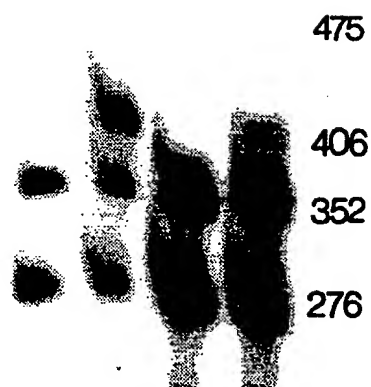
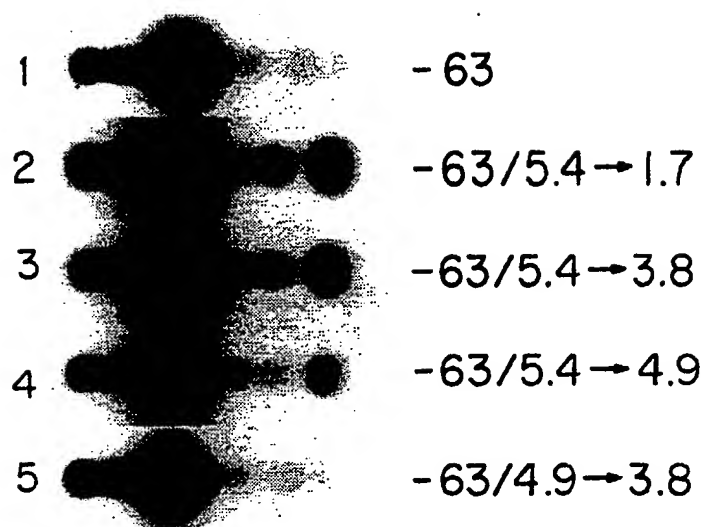


FIG. 3

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*FIG. 4*

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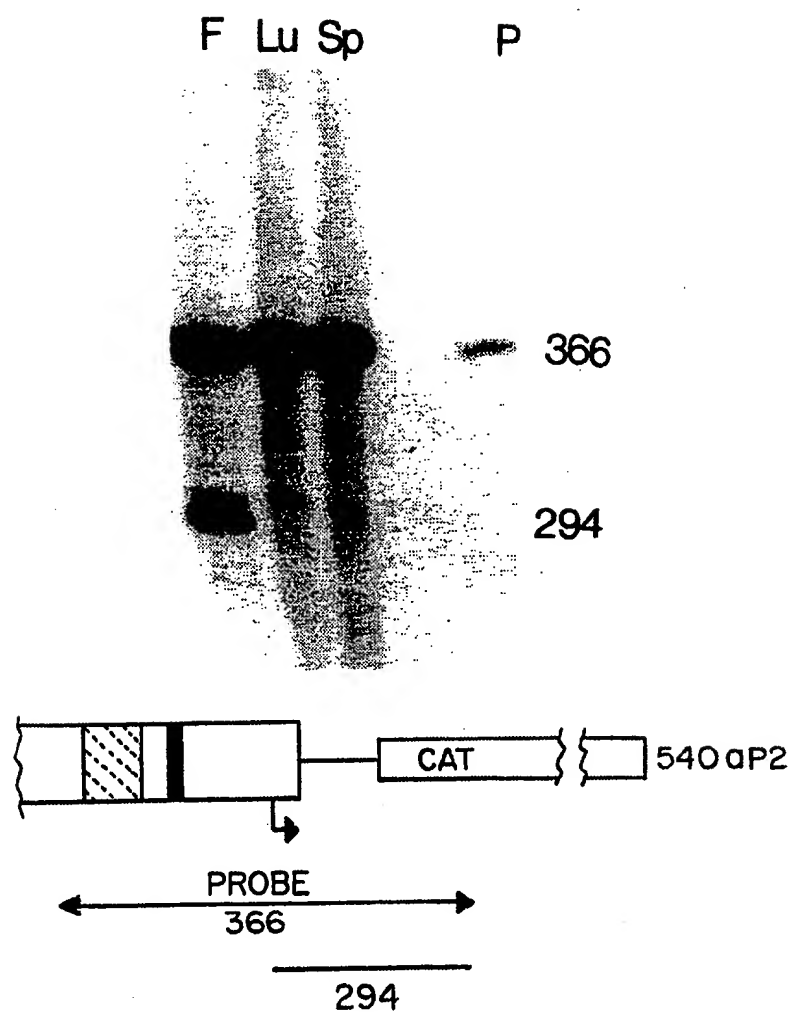
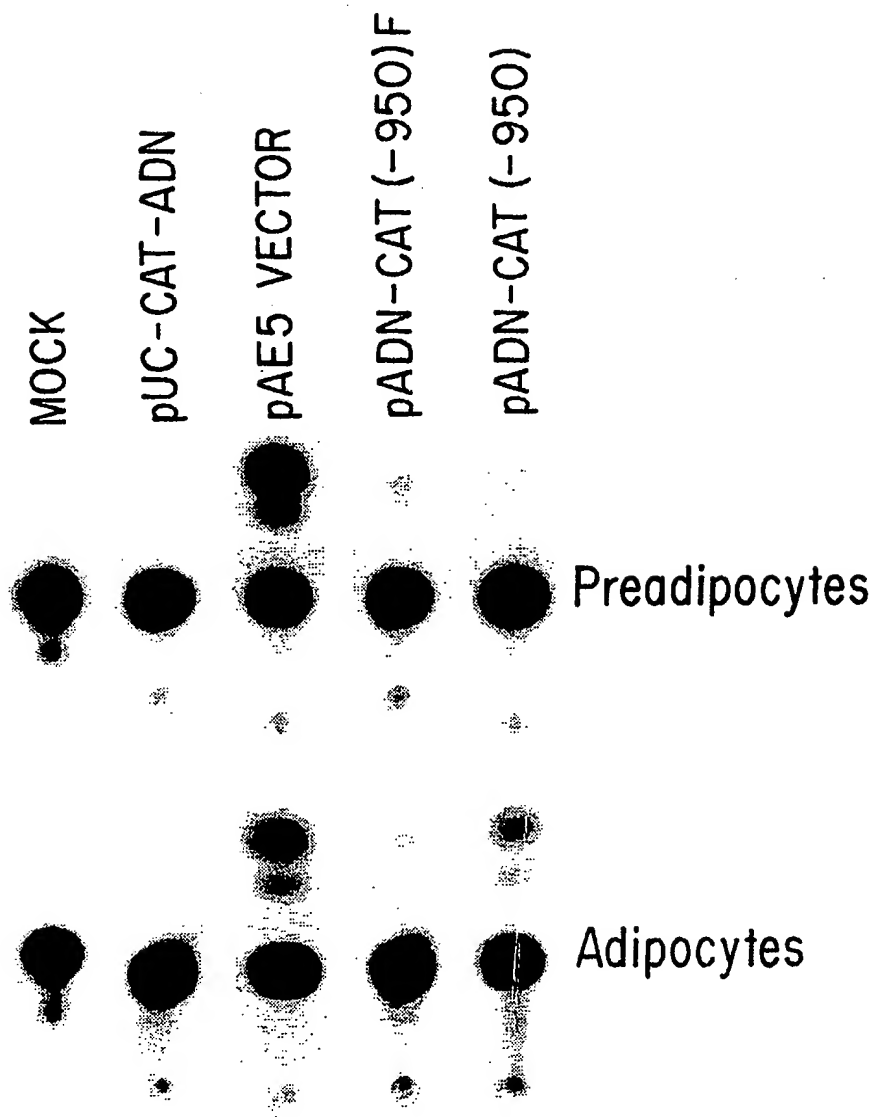


FIG. 5



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**FIG. 6****SUBSTITUTE SHEET**

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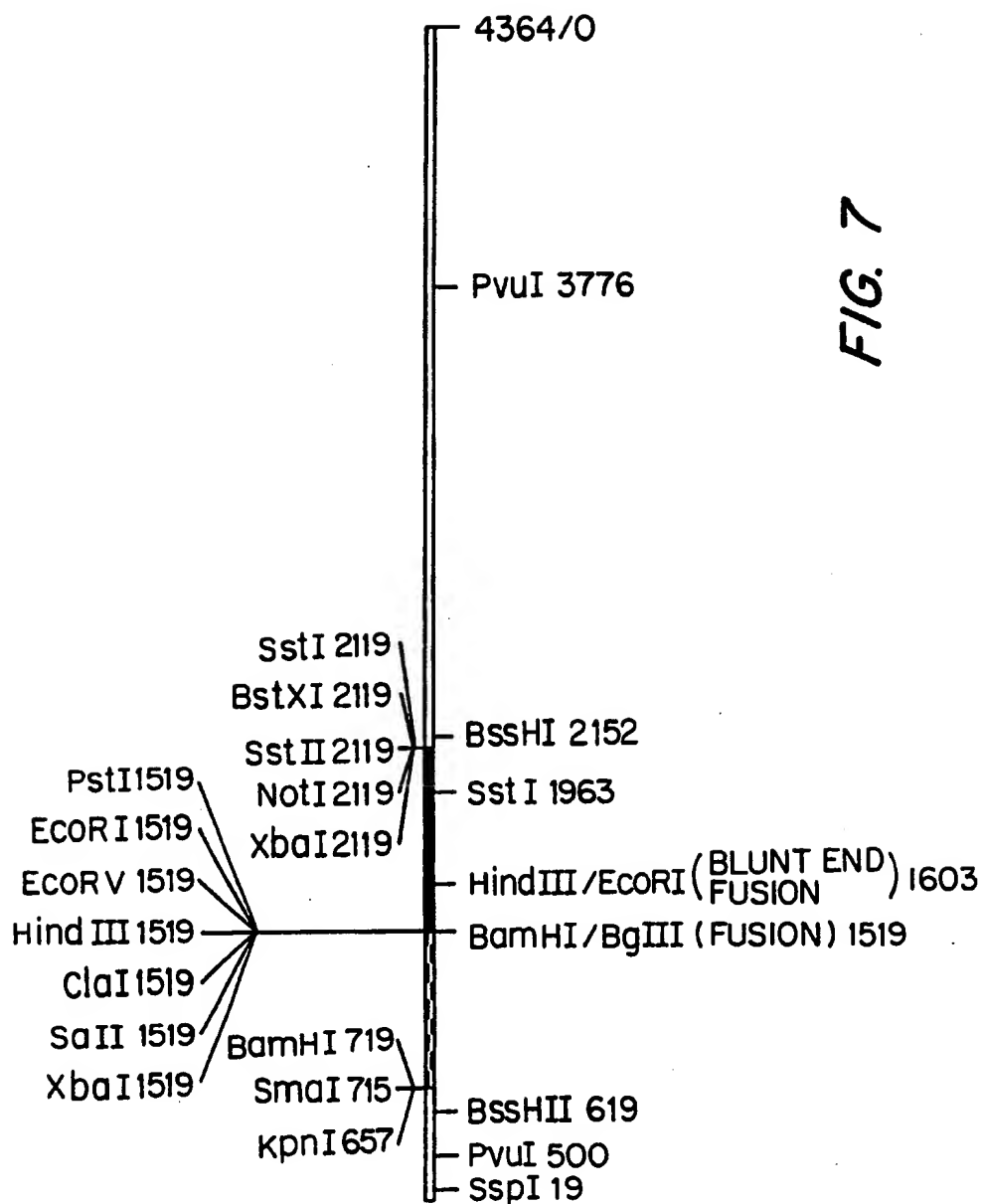


FIG. 7

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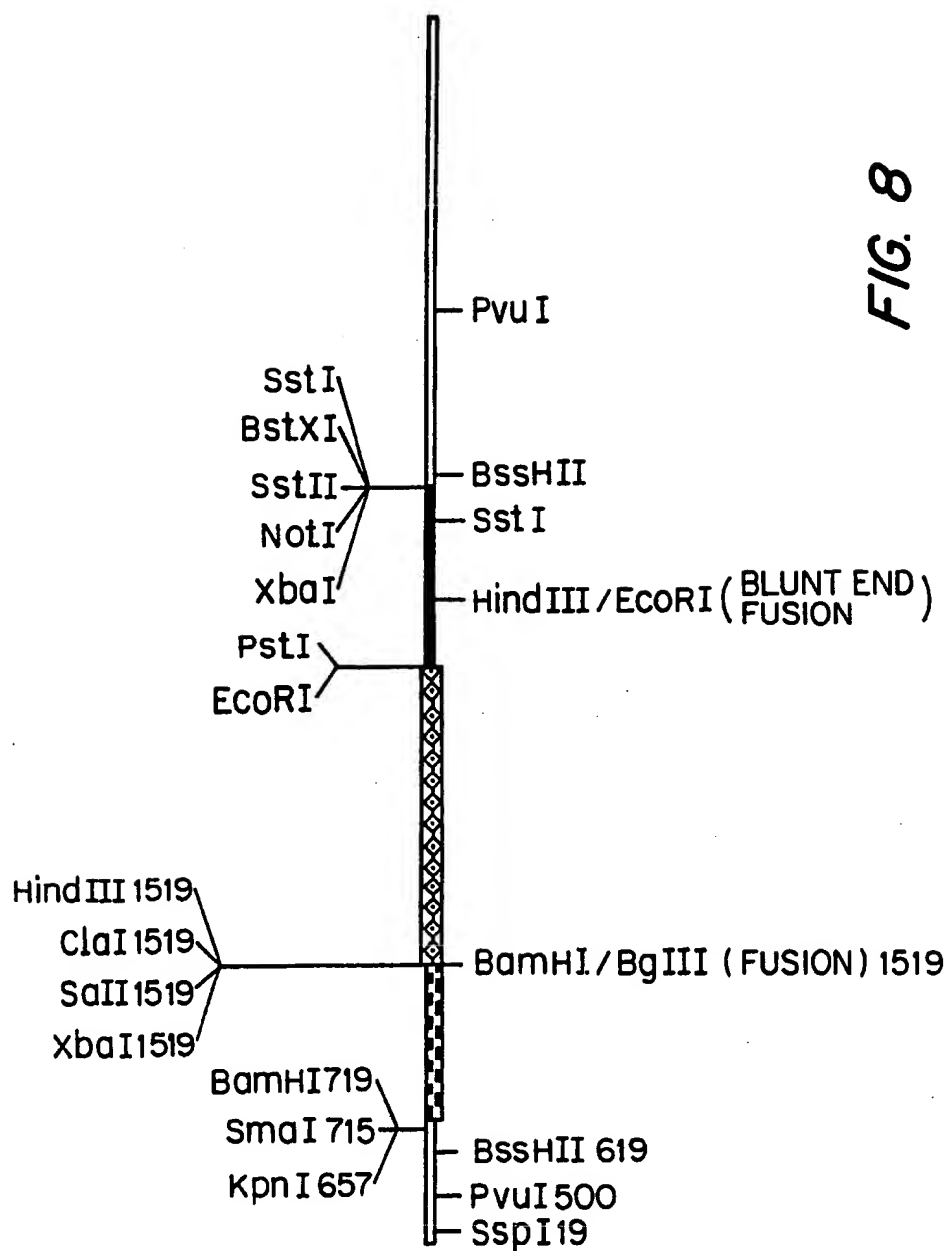
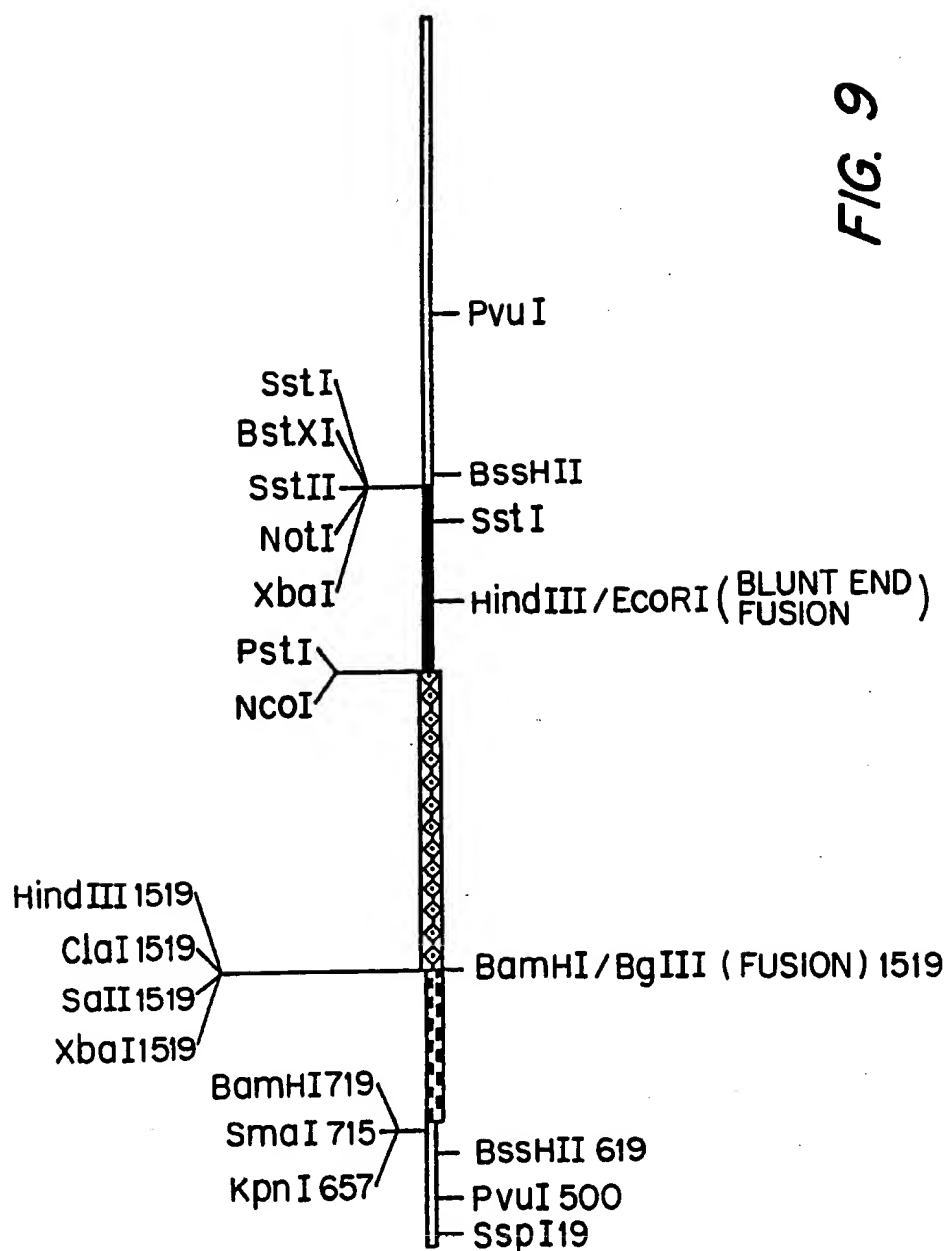


FIG. 8

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## INTERNATIONAL SEARCH REPORT

PCT/US91/06989

International Application No.

<b>I. CLASSIFICATION OF SUBJECT MATTER</b> (If several classification symbols apply, indicate all) *		
According to International Patent Classification (IPC) or to both National Classification and IPC U.S.Cl.: 536/27; 800/2 IPC(5): C07H 21/04		
<b>II. FIELDS SEARCHED</b>		
Minimum Documentation Searched *		
Classification System	Classification Symbols	
U.S.Cl.:	536/27; 800/2; 435/226; 935/34, 70	
Documentation Searched other than Minimum Documentation to the extent that such documents are included in the fields searched *		
APS, MEDLINE, BIOTECH ABS, AGRICOLA GENBANK search terms: expression, Lipogenesis, Adipose, Obesity, enhancer (sequence or element), promoter, gene, Adipocyte, transgenic, lipolysis		
<b>III. DOCUMENTS CONSIDERED TO BE RELEVANT *</b>		
Category *	Citation of Document, ** with indication, where appropriate, of the relevant passages **	Relevant to Claim No. **
A	Journal of Animal Sciences, Volume 67, Issued 1989, Gaskins et al. "Regulation of Gene Expression during Adipocyte Differentiation: A Review", pages 2263- 2272, see entire document.	1-8, 17-27
A	New Scientist, Volume 7, Issued July 1988, Wilmut, et al., "A Revolution in Animal Breeding", pages 56-59, see entire document.	17-25
Y	Journal of Biological Chemistry, Volume 261, No. 23, Issued 15 August 1986, Phillips et al., "The Nucleotide Sequence of Three Genes Participating in the Adipose Differentiation of 3T3 Cells," pages 10821-10827, see entire document.	1-27
Y	Proceedings of the National Academy of Sciences, Vol. 90, Issued October 1989, Platt et al., "Obesity-linked regulation of the adipon gene promoter in transgenic mice", pages 7490-7494, see entire document.	1-27
Y	Proceedings of the National Academy of Sciences, Vol. 83, Issued June 1986, Hunt et al., "Adipocyte P2 gene: Developmental expression and homology of 5'-flanking sequences among fat-specific genes", pages 3786-3790, see entire document.	1-27
<p>* Special categories of cited documents: **</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"Δ" document member of the same patent family</p>		
<b>IV. CERTIFICATION</b>		
Date of the Actual Completion of the International Search		Date of Mailing of this International Search Report
17 December 1991		07 JAN 1992
International Searching Authority		Signature of Authorized Officer
ISA/US		G. Bugajski

## FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE<sup>1</sup>

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers \_\_\_\_\_, because they relate to subject matter <sup>12</sup> not required to be searched by this Authority, namely:
  
2. ☐ Claim numbers \_\_\_\_\_, because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out <sup>12</sup>, specifically:
  
3. ☐ Claim numbers \_\_\_\_\_, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING<sup>1</sup>

This International Searching Authority found multiple inventions in this international application as follows:

I Claims 1-7, 26 & 27, drawn to DNA sequences, classified in class 536, subclass 27.

II Claims 8-25, drawn to transgenic animals & a method of their production classified in class 800, subclass 2

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application. (Telephone practice)
2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:
  
3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:
  
4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

## Remark on Protest

- ☐ The additional search fees were accompanied by applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

Attachment to PCT Telephone Memorandum  
PCT/US91/06989

I. Claims 1-7, 26 and 27, drawn to DNA sequences.  
Class 536, subclass 27.

II. Claims 8-25, drawn to transgenic animals and a method for  
their production. Class 800, subclass 2.

The claims of groups I and II are drawn to distinct products and have a separate status in the art as shown by their different classification. PCT Rules 13.1 and 13.2 do not provide for multiple distinct products within a single general inventive concept.